DOES NOT CIRCULATE

UNIVERSITY OF MICHIGAN

MAY 26 1954

MEDICAL LIBRARY

VOLUME 94 NUMBER 4

BULLETIN of the JOHNS HOPKINS HOSPITAL

THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL

SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY

April, 1954

The Multiplicity of Plasmin Inhibitors in Human Serum Demonstrated by the Effect of Primary Amino Compounds.
OSCAR D. RATNOFF, I. H. LEPOW AND LOUIS PILLEMER 169

A Simplified Method for the Routine Determination of Pregnanediol and Pregnanetriol in Urine.
ALFRED M. BONGIOVANNI AND GEORGE W. CLAYTON, JR. 180

Spectral Phonocardiography: Problems and Prospects in the Application of the Bell Sound Spectrograph to Phonocardiography.
VICTOR A. MCKUSICK, SAMUEL A. TALBOT AND GEORGE N. WEBB 187

Simultaneous Dye Dilution and Fick Determinations of Left and Right Ventricular Outputs in Dogs with End to End Anastomosis Between the Left Subclavian and Left Pulmonary Artery.
MORTON LEE PEARCE, HAROLD A. COLLINS, H. WILLIAM SCOTT, JR. AND ELLIOT V. NEWMAN 199

Studies on the Duration of Protection Afforded by Active Immunization Against Tetanus.
THOMAS B. TURNER, EDWARD S. STAFFORD AND LEON GOLDMAN 204

Abstracts of Papers Presented Before The Johns Hopkins Medical Society. February 8, 1954 and March 8, 1954 218

Book Reviews 222

PUBLISHED MONTHLY

THE JOHNS HOPKINS PRESS, Baltimore-18, Maryland

Made in United States of America

COPYRIGHT, 1954, BY THE JOHNS HOPKINS PRESS

UNIVERSITY OF MICHIGAN LIBRARIES

BULLETIN OF

THE JOHNS HOPKINS HOSPITAL

MANAGING EDITOR, PHILIP F. WAGLEY

ASSOCIATE MANAGING EDITOR, VERNON B. MOUNTCASTLE

EDITORIAL BOARD

E. Cowles Andrus — Charles R. Austrian — Henry T. Bahnson
Kenneth C. Blanchard — John Eager Howard
Arnold R. Rich

All correspondence relating to the publication of papers should be addressed to The Editor, Bulletin of The Johns Hopkins Hospital, Baltimore-5, Md.

Books for review should be sent to The Editor at the above address.

Correspondence concerning business matters and all remittances should be sent to The Johns Hopkins Press, Baltimore-18, Maryland, U. S. A.

Subscription price. In the United States and its possessions $8.00 per year. In Canada $8.25 per year; in foreign countries $8.50 per year. Single numbers, $1.00.

New subscriptions and renewals are entered to begin with the first issue of the current year. Subscribers in the United States are hereby notified that missing numbers cannot be replaced unless notification of loss is received in this office within 1 month following publication of the missing number; foreign subscribers 6 months.

Subscriptions in the British Empire except Canada may be sent to: Baillière, Tindall & Cox, 8 Henrietta St., Covent Garden, London, W.C. 2.

Manuscripts. Manuscripts should be typewritten, one side of page only, and double-spaced. Following the title should appear the author's name, department, and the university and/or hospital he represents. A list of references (bibliography) in numerical sequence should appear at the end of article. Preferred style for references is as follows: Author's last name, initials. Title of book or article, with first word and proper names capitalized only. Name of journal (abbreviate), date of publication, volume number: page number. The Bulletin is not responsible for loss of a manuscript in mail. Author should retain a duplicate copy.

Illustrations and Tables. Legends are required for Figures and descriptive headings for Tables. Use Arabic numerals to number Figures (Fig. 1); use Roman numerals to number Plates and Tables (Plate I). It should be indicated exactly where Figures and Tables are to appear in text; Plates all appear at end of article. All Figures should be drafted in black India ink for 50% reduction to 4½ inch width. Glossy photographs must be supplied for all Plates. Original illustrations will be returned to author with galley proofs.

Reprints. Twenty-five reprints, without covers, are furnished gratis for each article. Additional reprints should be ordered when galley proofs are returned.

THE JOHNS HOPKINS PRESS

BALTIMORE-18, MARYLAND, U. S. A.

Entered as second class matter at the Baltimore, Md., Post Office. Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917. Authorized on July 3, 1918.

THE MULTIPLICITY OF PLASMIN INHIBITORS IN HUMAN SERUM, DEMONSTRATED BY THE EFFECT OF PRIMARY AMINO COMPOUNDS*

OSCAR D. RATNOFF, I. H. LEPOW AND LOUIS PILLEMER

The Department of Medicine, Western Reserve University School of Medicine, The Institute of Pathology, Western Reserve University, and University Hospitals, Cleveland

Received for publication January 25, 1954

In an earlier study, it was observed that the addition of hydrazine to human serum reduced the ability of the serum to inhibit plasmin, a proteolytic enzyme of plasma which is active at or near neutrality (1). However, hydrazine-treated serum still retained considerable plasmin-inhibitory activity. It has also been demonstrated, by other methods, that there were at least two factors concerned with the inhibitory activity of serum (2). For this reason, the relationship of the hydrazine-sensitive inhibitor to other serum inhibitors of plasmin was investigated. It has been possible to demonstrate at least 3 inhibitors of plasmin in human serum, a heat-labile factor and two heat-stable inhibitors. The heat-stable inhibitors were separated further into those inactivated by hydrazine and those resistant to this compound. The possible relationship of the fourth component of complement to an inhibitor of plasmin was also tested.

NOMENCLATURE

The term *plasmin* (3) will be used to designate the enzyme or enzymes in plasma capable of digesting fibrinogen, fibrin, complement and certain other proteins *in vitro* at or near neutrality. This enzyme has also been called *fibrinolysin* (4) and *plasma proteolytic enzyme* (5). *Complement* will be designated C′ (6). Its fourth component, that portion of C′ which is stable at 56°C. for 30 minutes and which is inactivated by ammonia (7) and by hydrazine and primary amines (8), will be designated C′4 (6). The non-dialyzable fraction of serum soluble at pH 5.4 and ionic strength 0.02 at 0°C. will be referred to as R1, the fraction soluble under these conditions, R2, and serum treated with symosan to inactivate the third component at C′, R3 (9–11).

The *clot lysis time* is the length of time required for a visible fibrin clot to disappear. This was measured from the time the reagents were mixed, rather than from the time that clotting took place. This was a matter of convenience and did not alter the results since the clotting time was brief compared with the clot lysis time.

MATERIALS

Blood was drawn from normal human donors, allowed to stand overnight at 4°C., and its serum separated by centrifugation at room temperature. Serum used for C′ titrations was used within 24 hours after the blood was drawn. Serum used for plasmin studies was sometimes stored at −20°C. for as long as a week before use.

*This investigation was supported in part by a research grant (P.H.S. No. H1661C) from the National Institutes of Health, Public Health Service.

UNIVERSITY OF MICHIGAN LIBRARIES

Chloroform-activated bovine *plasmin*[1] (4) was dissolved in buffer (0.025M barbital in 0.125M sodium chloride, pH 7.5) in a concentration of 5 Loomis units (12.5 mg.) per ml. and stored at −20°C. until used.

One hundred mg. of bovine *fibrinogen*[2] (12) were dissolved in 20 ml. of buffer and the undissolved residue separated by filtration through Whatman No. 1 paper. This preparation contained 0.9 mg. sodium chloride for every 3.1 gm. protein. Assay of the filtrate demonstrated approximately 300 mg. coagulable protein per 100 ml. solution.

Bovine *thrombin*, Parke, Davis and Co., was dissolved in buffer in a concentration of 1000 National Institutes of Health (N.I.H.) units per ml. (13), and stored at −20°C. until used. This preparation possessed appreciable inhibitory activity against plasmin.

Human *R1*, *R2* and *R3* were prepared by methods previously described (9, 10, 14).

Bovine, equine and human *antifibrinolysin*,[1] purified plasmin inhibitors, (15) were dissolved in buffer in a concentration of 6 per cent.

Hydrazine and the *amino compounds* tested were each dissolved in water in the concentrations indicated, and the pH adjusted with 0.1N NaOH (Table II). With the exception of hydrazine and ammonia, the solutions were freshly prepared.

In the "complement fixation" studies, the specific soluble substance of the Type III pneumococcus (S-III)[3] was dissolved in 0.15M sodium chloride in a stock concentration of 0.1 per cent, and antipneumococcus rabbit serum[3] was diluted in 0.15M sodium chloride to contain 1 mg. of antibody nitrogen per ml.

METHODS

The *plasmin-inhibitory activity* of serum or its fractions was measured by a fibrinolytic method. Three-tenths ml. of serum, treated serum, or serum fractions, serially diluted with buffer, were incubated with an equal volume of plasmin at 37°C. for 10 minutes. The mixtures were then transferred to an ice bath. Three-tenths ml. of fibrinogen solution and 0.1 ml. of thrombin solution were added in rapid succession. The mixtures were then incubated at 37°C. and observed for fibrinolysis. In most experiments, the concentration of plasmin after all dilutions was 0.375 units per ml. In each experiment, the rate of fibrinolysis was compared with the rate of fibrinolysis by the same amount of plasmin in the absence of the substance to be tested, and by one half this amount of plasmin. Thus it was possible to determine by interpolation the concentration of the test substance which resulted in 50 per cent inhibition of plasmin. In most experiments the clots were observed for lysis for a period of 90 minutes.

The effect of various compounds upon the plasmin-inhibitory activity of serum was measured in the following manner. Two ml. aliquots of serum were mixed with 0.5 ml. of an aqueous solution of the substance to be tested, and 0.5 ml. of buffer. The pH was adjusted when necessary with 0.1N sodium hydroxide. This mixture was then incubated at 37°C. for one hour, the pH readjusted to 7.5–7.9 with 0.1N hydrochloric acid, and the serum then diluted serially with buffer. The plasmin-inhibitory activity of the diluted serums were then tested in the manner described. In any one experiment, no more than 3 or 4 compounds were tested simultaneously, since it was difficult to achieve accuracy with a greater number.

The titer of C′4 in samples of serum or its fractions was measured in a sheep erythrocyte hemolytic system by methods previously described (1, 9, 14, 16). The 50 per cent hemolytic end point was used in all titrations.

---

[1] Obtained through the courtesy of Dr. E. C. Loomis of Parke, Davis & Co.

[2] Obtained through the courtesy of Dr. Walter Seegers.

[3] Obtained through the courtesy of Lederle Laboratories Division, American Cyanamid Co.

Chloroform-treated serum was prepared by mixing serum with one-tenth its volume of chloroform in a glass-stoppered graduate. After ten minutes, the chloroform was separated by centrifugation at 2500 rpm for ten minutes at room temperature. Traces of chloroform in the supernatant serum were then removed by aeration.

"Complement-fixed" serum was prepared by treatment of fresh serum with anti-pneumococcic rabbit serum and S-III; 0.2 mg. of antibody nitrogen and 0.008 mg. of S-III were employed per ml. of serum. After incubation for 1 hour at 25°, the mixture was centrifuged and the clear supernatant retained at 0° for use within several hours. A control was prepared in exactly the same manner, except that S-III was omitted.

RESULTS

(*1*) *The effect of amino compounds on the plasmin inhibitory activity of serum*

Human serum was incubated with various amino compounds and the inhibitory activity of the mixtures was then tested against bovine plasmin. Serum treated with hydrazine had less than half the inhibitory activity of untreated serum or serum treated with hydroxylamine (Table I). Experiments were performed to determine the mechanism by which hydrazine acted. A freshly prepared mixture of hydrazine and plasmin had the same fibrinolytic activity as the plasmin alone; if the mixture was first allowed to react alone for 30 minutes at 37°C., it was less fibrinolytic than untreated plasmin. These experiments indicated that the effect of hydrazine which has been described was upon the serum itself and not upon either plasmin or the fibrin clot. Furthermore, the addition of hydrazine to serum did not induce measurable spontaneous fibrinolytic activity when tested under the same conditions used to determine the serum's inhibitory activity. One may conclude that the action of hydrazine was to reduce the inhibitory activity of the serum against active bovine

TABLE I

*The Influence of Hydrazine and Hydroxylamine on the Plasmin-Inhibitory Activity of Serum**

INITIAL CONC. OF SERUM	INITIAL CONC. OF PLASMIN	CLOT LYSIS TIME			
		Serum + NaCl	Serum + Hydrazine	Serum + Hydroxylamine	Buffer†
	units/ml.	*min.*	*min.*	*min.*	*min.*
1/4	2.5	∞	31	∞	—
1/8	"	∞	14	∞	—
1/16	"	22	9	17	—
1/32	"	9	9	9	—
1/64	"	9	7	8	—
1/128	"	7	7	7	—
0	"	—	—	—	7
0	1.25	—	—	—	11

* The serum was incubated for one hour at 37°C. with one-fourth its volume of 0.15M NaCl, 0.16M hydrazine, or 0.15M hydroxylamine and one-fourth its volume of buffer. The serum was then diluted serially with buffer, and its plasmin-inhibitory activity tested.

† Substituted for serum.

TABLE II

The Effect of Various Compounds Upon the Plasmin-Inhibitory Activity of Serum

REAGENT	FORMULA	MOLARITY*	pH†	INACTIVATION OF C′4‡	INACTIVATION OF PLASMIN INHIBITOR
Ammonia	NH_3	0.027M	9.5	+	+
Methylamine	CH_3NH_2	0.02M	7.5–8.0	+	+
Dimethylamine	$(CH_3)_2NH$	0.12M	7.5–8.0	−	−
Ethylamine	CH_3CH_2NH	0.04M	7.5–8.0	+	+
Hydrazine	NH_2NH_2	0.027M	7.5–8.0	+	+
Glycine	$COOHCH_2NH_2$	0.17M	7.5–8.0	−	−
Semicarbazide	$NH_2CONHNH_2$	0.09M	7.5–8.0	−	−
Hydroxylamine	$HONH_2$	0.03M	7.5–8.0	−	−
α Methylhydroxylamine	CH_3ONH_2	0.027M	8.5–9.5	+	+
Methenamine	$(CH_2)_6N$	0.16M	7.5–8.0	−	−

* After mixture with serum.

† pH of mixture of serum and reagent during preliminary incubation at 37°C.

‡ From Pillemer, L., Seifter, J. and Ecker, E. E. (8).

TABLE III

*The Effect of Alkalinity and of α Methylhydroxylamine on the Plasmin-Inhibitory Activity of Serum**

INITIAL CONC. OF SERUM	INIT. CONC. OF PLASMIN	CLOT LYSIS TIME			
		Serum @ pH 7.95†	Serum @ pH 9.50†	Serum + α methylhydroxylamine at pH 9.48†	Buffer‡
	units/ml.	min.	min.	min.	min.
1/4	1.25	∞	∞	35	—
1/8	1.25	∞	29	11	—
1/16	1.25	19	15	10	—
1/32	1.25	7	7	7	—
1/64	1.25	6	6	6	—
0	1.25	—	—	—	5
0	0.625	—	—	—	9

* The serum was incubated for one hour at 37°C. with or without one-fourth its volume of 0.16M α methylhydroxylamine at the pH indicated. All the samples were then adjusted to pH 7.95 and their inhibitory activity against plasmin determined.

† The C′4 titer of serum after incubation at pH 7.95 was 240 units/ml., at pH 9.50, 160 units/ml., and, after incubation with α methylhydroxylamine at pH 9.48, zero.

‡ Substituted for serum.

plasmin. The effect of other, similar compounds is listed in Table II. Ammonia and α methylhydroxylamine decreased the inhibitory activity of serum only in alkaline solution. The addition of weak sodium hydroxide to serum also decreased its inhibitory activity slightly, but the action of ammonia and of α methylhydroxylamine could not be accounted for merely on the basis of a change in pH (Table III). The effect of these compounds upon the plasmin inhibitors paralleled their effect upon C′4.

TABLE IV

*The Influence of Heat and of Hydrazine on the Plasmin-Inhibitory Activity of Serum**

INITIAL CONC. OF SERUM	INITIAL CONC. OF PLASMIN	CLOT LYSIS TIME				
		Unheated serum + NaCl	Unheated serum + hydrazine	Heated serum + NaCl	Heated serum + hydrazine	Buffer†
	units/ml.	*min.*	*min.*	*min.*	*min.*	*min.*
1/2	1.25	∞	∞	∞	45	—
1/4	1.25	∞	120	∞	22	—
1/8	1.25	∞	12	100	12	—
1/16	1.25	36	8	16	5	—
1/32	1.25	10	5	8	5	—
1/64	1.25	7	5	6	4	—
0	1.25	—	—	—	—	4
0	0.625	—	—	—	—	8

* Unheated serum, or serum which had been heated at 56°C. for 30 minutes was incubated for one hour at 37°C. with one-fourth its volume of 0.15M NaCl or 0.16M hydrazine, and its plasmin-inhibitory activity was then tested. Heating the serum at 56°C. for 60 minutes instead of 30 did not alter the results recorded.

† Substituted for serum.

The effect of various concentrations of hydrazine on the serum plasmin inhibitors was studied. A slight decrease in inhibitory activity occurred when serum was incubated for 1 hour at 37°C. with hydrazine in a final concentration of 0.027M, the lowest concentration tested. A maximal effect was noted at a final concentration of 0.019M; no further effect was observed at 0.06M.

(2) *The effect of heat and of chloroform upon the plasmin inhibitory activity of serum*

Heating serum at 56°C. for 30 minutes decreased its ability to inhibit plasmin; an additional 75 minutes produced no further change (Table IV). Hydrazine was equally effective in decreasing plasmin-inhibitory activity in heated and unheated serum. These experiments indicate that hydrazine and gentle heating alter different components of the plasmin inhibitors of serum.

Serum treated successively with heat and hydrazine still possessed some plasmin inhibitory activity (Table IV), even though the time of heating and concentration of hydrazine was apparently sufficient to produce a maximal effect. For this reason it appeared that the plasmin inhibitory activity of serum is composed of at least three different entities.

It has long been known that the mixture of serum and chloroform results in the development of spontaneous proteolytic activity. This has been attributed at least in part to the destruction of plasmin inhibitors by chloroform. Chloroform-treated serum had about half the plasmin-inhibitory activity of untreated serum (Table V). Heating chloroform-treated serum to 56°C. resulted in an additional decrease in its inhibitory activity, and conversely, the inhibitory

TABLE V

*The Influence of Chloroform on the Plasmin-Inhibitory Activity of Serum**

INIT. CONC. OF SERUM	INIT. CONC. OF PLASMIN	CLOT LYSIS TIME		
		Serum	$CHCl_3$-treated serum	Buffer†
	units/ml.	*min.*	*min.*	*min.*
1/2	1.25	∞	∞	—
1/4	1.25	∞	165	—
1/8	1.25	∞	21	—
1/16	1.25	18	8	—
1/32	1.25	6	4	—
1/64	1.25	4	4	—
0	1.25	—	—	4
0	0.62	—	—	8

* Untreated serum or serum which had been shaken for 10 minutes with chloroform was diluted serially with buffer and plasmin inhibition then measured.

† Substituted for serum.

activity of heated serum was further reduced by treatment with chloroform (Table VI). Hydrazine decreased the inhibitory activity of serum treated successively with heat and chloroform; about 1/8 of the original plasmin inhibitory activity remained after these procedures.

(3) *The effect of hydrazine on purified plasmin inhibitors*

Two ml. portions of 6 per cent solutions of purified human, equine and bovine plasmin inhibitors were incubated at 37°C. for one hour with 0.5 ml. buffer and either 0.5 ml. of 0.16M hydrazine or 0.15M sodium chloride solutions. The solutions were then diluted serially with buffer and their protease inhibitory activities tested. Hydrazine had no demonstrable influence upon these inhibitors. A parallel study of the C′4 activity of the 3 inhibitors revealed that they lacked this component of C′.

(4) *The differentiation of C′4 and the plasmin inhibitory properties of serum*

In the experiments recorded thus far, heat, pH, and certain amino compounds affected C′4 activity and the plasmin inhibitors of serum in the same manner. The possibility that C′4 was one of the inhibitors of plasmin was therefore studied. R1, R2, and R3 were incubated for one hour with solutions of hydrazine or sodium chloride, and the C′4 titer and plasmin inhibitory activities of the fractions were then tested (Table VII). The inactivation of C′4 in R1 and R3 paralleled the inactivation of plasmin inhibitor. The preparation of R2 employed was devoid of C′4 activity after incubation with sodium chloride, and at the same time was unaffected by hydrazine.

However, it did appear possible to differentiate C′4 activity and plasmin inhibitory activity. Treating serum with chloroform removed its C′4 activity,

TABLE VI

*The Influence of Heat, Chloroform and Hydrazine on the Plasmin-Inhibitory Activity of Serum**

INIT. CONC. OF SERUM	INIT. CONC. OF PLASMIN	CLOT LYSIS TIME				
		Serum†	Heated serum†	Heated serum CHCl$_3$-treated†	Heated serum CHCl-treated, + hydrazine†	Buffer‡
	units/ml.	*min.*	*min.*	*min.*	*min.*	*min.*
1/2	1.0	∞	∞	∞	48	—
1/4	1.0	∞	∞	∞	34	—
1/8	1.0	∞	∞	∞	15	—
1/16	1.0	∞	45	34	10	—
1/32	1.0	26	16	14	7	—
1/64	1.0	15	9	9	7	—
0	1.0	—	—	—	—	7½
0	0.5	—	—	—	—	15

* Successively, serum was heated at 56°C. for one hour, shaken with chloroform for 10 minutes, and incubated for one hour at 37°C. with one-fourth volume of 0.16M hydrazine. Samples of the unheated, heated and chloroform-treated serums were also incubated for one hour at 37°C. with one-fourth volume of 0.15M sodium chloride. The serums were then diluted serially with buffer, and their plasmin-inhibitory activities and C′4 titers were measured.

† The C′4 titer of untreated serum was 640 units/ml., of heated serum, 80 units/ml., and of heated, chloroform-treated serum, 0 units/ml.

‡ Substituted for serum.

TABLE VII

*The Influence of Hydrazine on the Plasmin-Inhibitory Activity of the R1, R2, and R3 Fractions of Serum**

INIT. CONC. OF SERUM	INIT. CONC. OF PLASMIN	CLOT LYSIS TIME						
		R1 + NaCl†	R1 + hydrazine†	R2 + NaCl†	R2 + hydrazine†	R3 + NaCl†	R3 + hydrazine†	Buffer‡
	units/ml.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1/2	1.25	∞	∞	10½	9½	∞	∞	—
1/4	1.25	∞	21	10½	9½	∞	∞	—
1/8	1.25	∞	9	9	9½	∞	20	—
1/16	1.25	15	8½	9	9	20	9	—
1/32	1.25	8½	6½	7½	6½	8	7	—
1/64	1.25	6	5½	6½	5½	6	6	—
0	1.25	—	—	—	—	—	—	5
0	0.62	—	—	—	—	—	—	10½

* R1, R2 and R3 were incubated for one hour at 37°C. with one-fourth volume of 0.15M sodium chloride or 0.16M hydrazine. They were then diluted serially with buffer and their plasmin-inhibitory and C′4 activities tested.

† The C′4 titer of R1 + NaCl was 240 units/ml., R1 + hydrazine, 10 units/ml., and R3 + NaCl was 480 units/ml. The C′4 titer of R2 + NaCl or hydrazine, and R3 + hydrazine was zero.

‡ Substituted for serum.

TABLE VIII

*The Influence of Antigen-Antibody Reactions on the Plasmin-Inhibitory Activity of Serum**

INIT. CONC. OF SERUM	INIT. CONC. OF PLASMIN	CLOT LYSIS TIME				
		Serum + antibody		Serum + antigen + antibody		Buffer‡
		+ NaCl†	+ hydrazine†	+ NaCl†	+ hydrazine†	
	units/ml.	min.	min.	min.	min.	min.
1/4	1.25	∞	∞	∞	∞	—
1/8	1.25	∞	14	∞	14	—
1/16	1.25	16	7	12	7	—
1/32	1.25	8	5	8	5	—
0	1.25	—	—	—	—	5
0	0.62	—	—	—	—	9½

* Serum was incubated with antibody alone or with antigen and antibody. The inhibitory activity of the serums was then tested after incubation for one hour at 37°C. with 0.15M sodium chloride or 0.16M hydrazine.

† The C′4 titer of serum, antibody and NaCl alone was 320 units/ml. The C′4 titers of serum, antibody and hydrazine, and serum, antigen, antibody and NaCl or hydrazine were zero.

‡ Substituted for serum.

but a plasmin inhibitor susceptible to hydrazine was still present (Table VI). When C′4 activity was removed from serum by "fixation" upon an immune precipitate, the total amount of plasmin inhibitor in the serum and the amount sensitive to hydrazine were unaltered (Table VIII).

A third distinction between C′4 activity and plasmin inhibitory activity was achieved by incubating serum at 37°C. for one hour with bovine plasmin in final concentration of 1.0 units per ml. This procedure inactivates C′4 (1). Nonetheless, the plasmin-inhibitory titer of the serum was equal in the treated and untreated serums.

DISCUSSION

Human serum possesses considerable inhibitory activity against plasmin. In a study of the factors controlling the rate of fibrinolysis, it was reported that there are at least two inhibitors of plasmin in plasma or serum (2). One component deteriorated during incubation of the plasma at 37°C., whereas the other was stable under these conditions. The stable component, in turn, was partially inactivated by heating the serum at 56°C. for half an hour (17).

The present experiments confirm the view that the ability of serum to inhibit plasmin is not confined to a single component. Heat-labile and heat-stable inhibitors for this enzyme could be recognized by heating serum at 56°C. for 30 minutes. The heat-stable inhibitors could be separated further into factors which were inactivated by hydrazine and those which were not.

Ammonia, methylamine, ethylamine and α methylhydroxylamine also de-

creased the plasmin-inhibitory activity of serum. These compounds contain highly reactive NH_2 groups, and are all capable of reacting with carbonyl groups. Primary amino groups were apparently necessary for the activity of these compounds, since secondary amines and methenamine were without effect. Moreover, the substituent group which replaced the third hydrogen of ammonia to form the amino compound was of importance. Glycine, semicarbazide and hydroxylamine were all without influence on the inhibition of plasmin by serum. The effect of these various amino compounds suggests that this portion of the serum plasmin inhibitory activity may contain a carbohydrate moiety.

Chloroform diminishes the protease inhibitory activity of serum (18). In the present experiments, shaking serum with chloroform for 10 minutes decreased its inhibitory activity 50 per cent or less. In contrast, Christensen (19) reported that the trypsin-inhibitory activity of serum was reduced to 10 per cent of its original value by shaking with chloroform, an observation which it has been possible to confirm (17). However, no evidence has been presented that chloroform acted upon any specific component of the plasmin inhibitors. The complexity of the problem is indicated by the fact that serum treated successively by heat, chloroform and hydrazine was still able to inhibit plasmin to a slight degree.

There have been many attempts to identify the components of C′ with components of the blood clotting or proteolytic systems. The evidence presented in this paper at first suggested the identity of C′4 and the heat-stable plasmin inhibitor of serum. However, experiments were performed which appeared to separate C′4 and plasmin inhibitory activity. Geiger (20) too has shown that immune aggregates do not affect the plasmin inhibitors of serum. However, none of the experiments exclude the possibility that C′4 and the hydrazine-sensitive inhibitor represent activities of different parts of the same molecule.

Loomis and his associates (15) prepared fractions of bovine, equine and human serum relatively rich in inhibitors of bovine plasmin. These fractions were separated by procedures which yield fractions rich in α globulins. In human serum, C′4 is also associated with α globulins (21). It is of interest that hydrazine did not alter the plasmin inhibitory activity of these purified plasmin inhibitors. Hydrazine was also without effect upon the plasmin inhibitory properties of the R2 fraction of serum, that is, the fraction insoluble at pH 5.4 and low ionic strength. Work is in progress to attempt the isolation of the hydrazine-sensitive component of the serum inhibitors and clarify its relationship to the components of C′.

In any event, evidence has been presented for a multiplicity of serum inhibitors against plasmin. Data presented earlier concerning the inhibition of this enzyme by serum may require re-interpretation with this in mind.

SUMMARY

(1) Human serum appears to contain at least 3 inhibitors of plasmin. One is destroyed by heating serum at 56°C. for 30 minutes. One of the heat-stable plasmin inhibitors can be inactivated by ammonia, hydrazine and certain primary amines, but secondary amines and polar-substituted primary amines are ineffective. Serum which has been treated successively by heat and hydrazine still contains some plasmin-inhibitory activity, indicating the presence of a third component.

(2) Although C'4 and the hydrazine-sensitive plasmin inhibitor of serum resemble each other closely and are affected similarly by amino compounds, heat and alkali, it appears that the activities of these agents can be separated from each other.

This study would not have been possible without the technical assistance of Miss Joan Colopy and Miss Leona Wurz.

BIBLIOGRAPHY

1. Pillemer, L., Ratnoff, O. D., Blum, L. and Lepow, I. H.: The inactivation of complement and its components by plasmin. J. Exp. Med., 1953, **97:** 573.
2. Ratnoff, O. D.: Studies on a proteolytic enzyme in human plasma. III. Some factors controlling the rate of fibrinolysis. J. Exp. Med. 1948, **88:** 401.
3. Christensen, L. R. and MacLeod, C. M.: A proteolytic enzyme of serum: Characterization, activation and reaction with inhibitors. J. Gen. Physiol. 1945, **28:** 559.
4. Loomis, E. C., George, C., Jr., and Ryder, A.: Fibrinolysin: Nomenclature, unit, assay, preparation and properties. Arch. Biochem. 1947, **12:** 1.
5. Ratnoff, O. D.: Studies on a proteolytic enzyme in human plasma. I. The probable identity of the enzymes activated by chloroform and by filtrates of cultures of beta hemolytic streptococci. J. Exp. Med. 1948, **87:** 199.
6. Pillemer, L. and Ecker, E. E.: The terminology of the components of complement. Science 1941, **94:** 437.
7. Gordon, J., Whitehead, H. R. and Wormall, A.: The action of ammonia on complement. The fourth component. Biochem. J. 1926, **20:** 1028.
8. Pillemer, L., Seifter, J. and Ecker, E. E.: The effect of amino compounds on the fourth component of complement. J. Immunol. 1941, **40:** 89.
9. Bier, O. G., Leyton, G., Mayer, M. M. and Heidelberger, M.: A comparison of human and guinea pig complements and their component fractions. J. Exp. Med. 1945, **81:** 449.
10. Ecker, E. E., Pillemer, L. and Seifter, S.: Immunochemical studies on human serum. I. Human complement and its components. J. Immunol. 1943, **47:** 181.
11. Pillemer, L. and Ecker, E. E.: Anticomplementary factor in fresh yeast. J. Biol. Chem., 1941, **137:** 139.
12. Ware, A. G., Guest, M. M. and Seegers, W. H.: Fibrinogen: With special reference to its preparation and certain properties of the product. Arch. Biochem. 1947, **13:** 231.
13. Seegers, W. H. and Smith, H. P.: Factors which influence the activity of purified Thrombin. Am. J. Physiol., 1942, **137:** 348.

14. KABAT, E. A. AND MAYER, M. M.: *Experimental Immunochemistry*. C. C. Thomas, Springfield, Illinois, 1948.
15. LOOMIS, E. C., RYDER, A. AND GEORGE, C., JR.: Fibrinolysin and antifibrinolysin. Biochemical concentration of antifibrinolysin. Biochem., 1949, **20:** 444.
16. LEPOW, I. H., PILLEMER, L. AND RATNOFF, O. D.: The influence of calcium on the inactivation of human complement and its components by plasmin. J. Exp. Med., 1953, **98:** 277.
17. RATNOFF, O. D. Unpublished observations.
18. DELEZENNE, C. AND POZERSKI, E.: Action proteolyticque du serum sanguin prealablement traité par le chloroforme. Compt. rend. Soc. biol., 1903, **55:** 690.
19. CHRISTENSEN, L. R.: The activation of plasminogen by chloroform. J. Gen. Physiol. 1946, **30:** 149.
20. GEIGER, W. B.: Protease activation in immune reactions. J. Immunol., 1952, **68:** 11.
21. PILLEMER, L. Recent advances in the chemistry of complement. Chem. Rev., 1943, **33:** 1.

A SIMPLIFIED METHOD FOR THE ROUTINE DETERMINATION OF PREGNANEDIOL AND PREGNANETRIOL IN URINE[1]

ALFRED M. BONGIOVANNI AND GEORGE W. CLAYTON, JR.

With the technical assistance of BETTY LAWRENCE

Department of Pediatrics, The Johns Hopkins University School of Medicine

Received for publication January 26, 1954

Previous studies (1, 2) from this laboratory indicated the excretion of large quantities of 3-alpha-17-alpha-20-alpha-pregnanetriol (hereafter referred to as pregnanetriol) in the adrenogenital syndrome due to adrenocortical hyperplasia and their suppression by cortisone. Although earlier reports (3, 4) suggested the presence of large quantities of 3-alpha-20-alpha-pregnanediol (hereafter referred to as pregnanediol) in the urine of individuals with this syndrome, it appeared that most of the material so measured might be accounted for by the presence of pregnanetriol which exerts a relatively great influence on the reaction utilized for these determinations. It was shown that the method of Sommerville and Marrian (5) might be applied as a simple screening device for the presence of pregnanetriol provided that certain details were observed as described earlier (1). The latter method has continued to be useful but has certain objections discussed below, so that a simple chromatographic technique has been devised for the measurement of both pregnanediol and pregnanetriol in a single urine specimen. This has proved to be of great value in the diagnosis and treatment of patients with the adrenogenital syndrome due to adrenocortical hyperplasia. In addition, with this method, the results suggest that pregnanetriol is excreted normally in small amounts.

METHOD

A 50 ml. aliquot of a freshly collected 24-hour specimen of urine is incubated at 37°C. with 15,000 units glucuronidase[2] and 10 ml. of 0.5 M acetate buffer pH 4.5 for 24 hours. When high levels of steroid metabolites are expected only 25 ml. of urine with proportionately less enzyme and buffer are employed.

The urine is then extracted three times with one-third volume of benzene (thiophene-free, redistilled over sodium). The urine is discarded. The benzene is washed three times with 15 ml. of 1.0 N sodium hydroxide solution and three times with water. The benzene is now evaporated on a steam bath with a gentle air blast.

A column is prepared (11.0 mm. diameter) with 12 grams alumina[3] and benzene. The urine

[1] This work was made possible by a Grant-in-Aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[2] The glucuronidase was obtained from the Warner Institute, New York, N. Y.

[3] The alumina was purchased from the Harshaw Chemical Co., Elyria, Ohio.

residue is dissolved in 5.0–10.0 ml. benzene with the aid of gentle heat and applied to the column. Two extra washings of the residue with 3–4 ml. benzene are performed to insure quantitative transfer of the material to the column.

A simple three fraction elution with the following solutions is employed: (a) 100 ml. of 0.05 per cent ethanol in benzene (this may be discarded); (b) 150 ml. 2.0 per cent ethanol in benzene; (c) 100 ml. 10.0 per cent ethanol in benzene. The second fraction contains the pregnanediol and the last pregnanetriol. A 50 ml. aliquot of the second and third fraction is evaporated in a flask, with air to dryness. The remainder may be set aside for other studies. To the dry flasks is added 5.0 ml. ethanol which is then evaporated, to insure complete removal of the benzene. To each of the dry flasks is added 10.0 ml. concentrated sulfuric acid, with swirling, and the resultant chromogens are read in 20 minutes with the Beckman DU spectrophotometer at 325, 390, 425, 440 and 460 mu.

When the quantities of steroids are unusually large, it is necessary to dilute the solutions further with sulfuric acid. With experience, the need for this dilution may be determined by inspection.

The results are calculated by application of a correction factor according to the principles of Allen (6). For both compounds, the corrected reading at 425 mu is employed as follows:

$$\text{Corrected reading at 425 mu} = \text{OD at 425} - \frac{\text{OD 390} + \text{OD 460}}{2}$$

It has been found that the application of such a correction to various quantities of pure pregnanediol and pregnanetriol obeys Beer's law over a wide range, in spite of the fact that the true maximum for pregnanetriol in this region is 440 mu. From this reading the total quantity of each steroid present is calculated by use of a coefficient of extinction for each compound, corrected in the same fashion at 425 mu. The corrected coefficient[4] of extinction for pregnanetriol has been found to be 283 and that for pregnanediol 40. Repeated determinations of standards has yielded reasonably constant values so that these coefficients are generally used because of the difficulty in obtaining sufficient quantities of pure steroids as standards. The final estimation of the quantity of each compound per day must take into account the aliquot of total urine volume, the aliquot of the fraction from the column, and any further dilution with sulfuric acid.

RESULTS

The sulfuric acid chromogens of pregnanediol and pregnanetriol are shown in Figure 1. The relationships at the wavelengths utilized in the method are noteworthy. Pregnanetriol reveals a good peak at 425 mu, as does pregnanediol, but differs in its maximum which is 440 mu in this region. In addition, the true maximum for pregnanediol lies in the vicinity of 325 mu so that this should represent the highest reading for the second fraction while pregnanetriol reveals minimal light absorption below 400 mu. These relationships are applied to the results which should bear a resemblance to those of the pure compounds as a partial guarantee of relative purity of the residues from urine. The extinction coefficients ($\sum_{1.0\text{ cm.}}^{1.0\%}$) at various wavelengths of the pure compounds in concentrated sulfuric acid are given in Table I.

[4] $\sum_{1.0\text{ cm.}}^{1.0\%}$ (corrected at 425 as above).

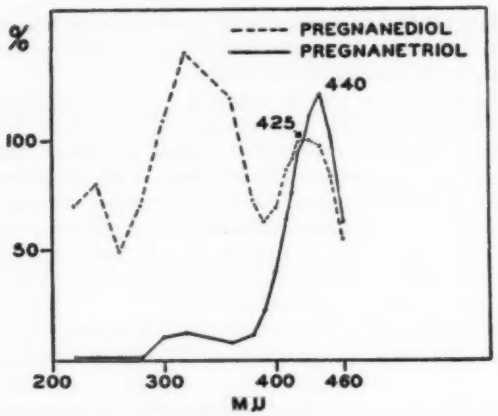


FIG. 1. The chromogens of pregnanediol and pregnanetriol in concentrated sulfuric acid have been read over a broad range, and the optical density at 425 mu has arbitrarily been indicated as 100 per cent in order to bring the differences into better focus.

TABLE I

Coefficients of Extinction $\left(\sum_{1\text{ cm.}}^{1\%}\right)$ *of Pregnanediol and Pregnanetriol at Various Wave Lengths*

Mu	325	390	425	440	460
Pregnanediol	259	91	114	108	65
Pregnanetriol	109	175	528	611	316

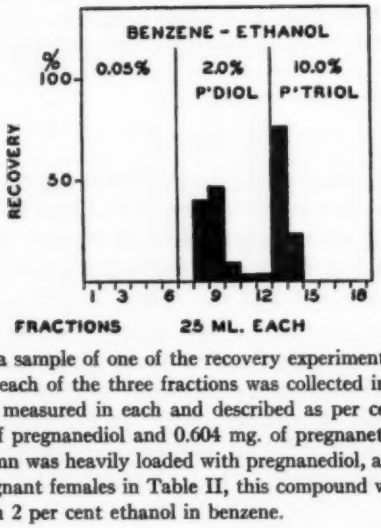


FIG. 2. Illustrated is a sample of one of the recovery experiments with the three eluates as described. However, each of the three fractions was collected in portions of 25 ml. and the quantity of steroid measured in each and described as per cent of the total. In this experiment 0.406 mg. of pregnanediol and 0.604 mg. of pregnanetriol were applied to the column. When the column was heavily loaded with pregnanediol, as indicated by the specimens of urine from pregnant females in Table II, this compound was entirely recovered in the second fraction with 2 per cent ethanol in benzene.

TABLE II

SUBJECT	SEX	AGE	DIAGNOSIS AND STATUS	PREGNANEDIOL	PREGNANETRIOL
				mg. per 24 hours	
AB	M	31	Normal	0.7	1.3
MG	M	29	Normal	0.9	0.3
MC	F	25	Normal	4.1	1.8
MFG	F	30	Pregnant, normal, 8 mo.	37.5	0.2
PV	F	30	Pregnant, normal, 6 mo.	16.4	0.3
			Adrenal Hyperplasia		
NW	F	16	Untreated	1.4	11.9
				.0	30.6
HW	F	30	Untreated	3.2	43.6
			Cortisone, 1 mo.	.0	.0
MB	F	34	Untreated	13.2	39.3
			ACTH 30 mg.	15.0	60.8
MC	M	3 mos.	Untreated	.0	3.0
				0.5	3.75
			Cortisone, 10 days	.0	0.2
WW	M	2	Untreated	2.5	10.5
			Cortisone, 10 days	.0	.0
			Cortisone, 3 wks.	0.6	.0
SB	F	10	Untreated	13.4	31.6
MD	F	1 mos.	(salt-losing)	.0	5.4
LL	F	1	Untreated	0.9	4.6
FF	F	3	Untreated	5.5	26.1
DD	F	12	Untreated	11.1	28.2
PD	F	14	Untreated	24.2	26.8
BT	F	4 mos.	Untreated	—	4.8
			Adrenal Hyperplasia (Girard Separation, Non-Ketonic)		
SB	(as above)			6.4	33.8
PD	(as above)			5.2	24.6

Figure 2 illustrates the results of a single recovery study with pure samples of pregnanediol and pregnanetriol. Other studies using 6.0 per cent ethanol instead of 10.0 per cent ethanol in benzene for collection of the pregnanetriol fraction indicated that much larger quantities of eluent were required for complete recovery and seemed to offer no advantages. However, it is known that pregnanetriol may be eluted with as little as 4.0 per cent ethanol in benzene if sufficient eluent is applied to the column.

Table II lists the results obtained with this method in normal adults, including pregnant females, and patients with the adrenogenital syndrome due to adrenocortical hyperplasia. The latter were studied before therapy and after suppressive treatment with cortisone. It may be seen that small quantities of pregnanetriol appear in the urines of normal adults. The levels excreted by

patients with the adrenogenital syndrome are greatly elevated and are highest in older individuals who had not been treated. The amount diminishes markedly on treatment with cortisone. The quantities of pregnanediol are uniformly less than those of pregnanetriol.

DISCUSSION

The method described has been an aid in the diagnosis and treatment of patients with the adrenogenital syndrome. The high levels of pregnanetriol excreted have helped confirm this diagnosis in patients in whom the excretion of 17-ketosteroids had not been initially as high as expected so that it was difficult to exclude the possibility of constitutional sexual precocity. The clearly elevated levels of pregnanetriol have shed light on the abnormalities of steroid synthesis in this disease, which has been discussed in detail elsewhere (2). They probably reflect an inability to convert 17-hydroxyprogesterone into compound F. Treatment with cortisone leads to a suppression of the abnormal activities of the adrenals since it replaces in a physiological manner an essential steroid which the diseased adrenal is unable to synthesize efficiently. The pituitary, previously overactive because of the low level of compound F, is now suppressed and the excretion of 17-ketosteroids and pregnanetriol falls. It seems that moderate quantities of pregnanediol may also be present but these are generally much smaller than the amounts of pregnanetriol. In addition, it appears that small quantities of pregnanetriol are also excreted by normal adults. This has recently been reported by Cox and Marrian (7) as well.

Although it has been stated that pregnanetriol is poorly soluble in benzene (8), large quantities of this steroid have been extracted with this solvent in the present and a previous study (1). Furthermore, with the aid of gentle heat it has always been possible to dissolve the residue of urines containing large quantities of pregnanetriol in small volumes of benzene for application to columns of alumina, from which relatively great amounts of the pure compound were subsequently eluted.

The screening procedure (1) for the detection of pregnanetriol previously described is based on the method of Sommerville and Marrian (5) for the estimation of pregnanediol. It was shown that by reading over a broad range of the visible spectrum, a peak was observed at 440 mu instead of 425 mu when large quantities of pregnanetriol were present. This remains a useful tool but the results were not as refined as those obtained with the method herein described for several reasons. With pure compounds, a number of studies indicated that at least 7 per cent of a mixture of the two steroids must consist of pregnanetriol before a peak at 440 mu might be expected. With crude urine residues, the percentage seemed to be much higher and very variable. For this reason, the small quantities present in normal urine are not detectable. Formulae devised from the coefficients of extinction of each compound at various wavelengths were

found to yield reliable results when measuring the quantities of each in a mixture of two pure samples in sulfuric acid. Unfortunately, these were grossly inaccurate when applied to measurements of urine residues of patients with the adrenogenital syndrome. Finally, although it was originally believed that pregnanetriol survived the procedure of Sommerville and Marrian for the determination of pregnanediol, this was subsequently found to be otherwise. Pure pregnanetriol submitted to the acid hydrolysis and heat of the latter method (9) and subsequently chromatographed on alumina, no longer eluted as did pregnanetriol. It was readily washed off the column with benzene and 0.1 per cent ethanol in benzene. However, the sulfuric acid chromogens remained identical with those of pregnanetriol so that the use of the method as a screening device was not invalidated. This can be explained by speculating that the acid and heat produced changes in the molecule similar to those caused by concentrated sulfuric acid itself. Zaffaroni (10) has in fact presented data which would support this thesis. The material eluted from the column after this treatment of pregnanetriol was examined by infra-red spectrophotometry and was found to be not pregnanetriol but an unidentified steroid.

Glucuronidase hydrolysis was selected in order to avoid the introduction of steroid artefacts such as those described. The system of elution has indicated that the pregnanetriol fraction is relatively pure on the basis of sulfuric acid chromogens closely resembling those of the pure compound and infra-red spectroscopy. It is this compound which has been of most interest in the adrenogenital syndrome and it was postulated (2) that its presence in large quantities indicated interference with the synthesis of 17-hydroxy-corticosterone in this condition. The pregnanediol eluate is expected to be contaminated by other steroids, particularly the 17-ketosteroids which have been shown to leave the column with similar or smaller concentrations of ethanol. However, the yield of 17-ketosteroids with glucuronidase hydroysis in this laboratory is generally less than 50 per cent of the total (acid hydrolysis); hence, the method employed has minimized such interference. It is, however, necessary to establish a relatively close correlation with the relationships at several wavelengths as shown in Table I in order to accept the material in the second fraction as pregnanediol with any degree of security. Obviously, a routine Girard separation of the urine residues with chromatography of the nonketonic fraction as described would overcome this objection. Although this has been done, it was not performed as a routine since the interest in this laboratory has centered on the excretion of pregnanetriol. When the Girard separation was employed (Table II), it became apparent that the values for pregnanediol in the urines of patients with the adrenogenital syndrome are considerably lower than those of pregnanetriol. This suggests that most of the levels of pregnanediol presented are high due to ketonic material in this fraction, hence a Girard separation is recommended for the accurate determination of this compound. The studies on normal adult

males did not reveal unduly elevated excretions of pregnanediol so that the ketonic steroids probably exert less influence on this fraction than in the adrenogenital syndrome. It is not essential to employ the Girard separation for the determination of pregnanetriol in the urine of patients with adrenal hyperplasia as a diagnostic aid or in following the therapeutic response to cortisone. The third fraction has been found to be relatively free of ketonic material as measured by the Zimmerman reaction and has yielded satisfactory infra-red spectra.

CONCLUSIONS

A method has been described for the measurement of pregnanediol and pregnanetriol in urine extracts. It has been a useful clinical tool in the diagnosis and treatment of the adrenogenital syndrome, which is characterized by the excretion of large quantities of pregnanetriol. It was found that moderate amounts of pregnanediol are present as well. Further study revealed the excretion of small quantities (0.2–1.8 mg.) of pregnanetriol by normal adults.

ACKNOWLEDGMENTS

The advice of Doctor Lawson Wilkins is gratefully acknowledged. The infra-red spectroscopy was performed through the kindness of Doctor Seymour Lieberman. Samples of pure pregnanetriol were generously supplied by Doctor Harold Mason.

REFERENCES

1. Bongiovanni, A. M.: The detection of pregnanediol and pregnanetriol in the urine of patients with adrenal hyperplasia. Suppression with cortisone. Bull. Johns Hopkins Hosp. 1953, **92**: 244.
2. Bongiovanni, A. M., Eberlein, W. R. and Cara, J.: Studies on the metabolism of adrenal steroids in the adrenogenital syndrome. J. Clin. Endocrinol. & Metab., in press.
3. Venning, E. H., Weil, P. G. and Browne, J. S.: Excretion of sodium pregnanediol glucuronidate in the adrenogenital syndrome. J. Biol. Chem. 1939 **128**: CVII.
4. Genitis, V. E. and Bronstein, I. P.: Pregnanediol excretion in female pseudohermaphroditism. J. Am. Med. Assn. 1942, **119**: 704.
5. Sommerville, I. F. and Marrian, G. F.: Rapid determination of pregnanediol. Lancet 1948, **2**: 89.
6. Allen, W. M.: A simple method for analyzing complicated absorption curves, of use in the colorimetric determination of urinary steroids. J. Clin. Endocrinol. 1950, **10**: 71.
7. Cox, R. I. and Marrian, G. F.: The solution of pregnane-3-alpha-17-alpha-20-alpha-triol from the urine of normal men. Biochem. J. 1953, **54**: 353.
8. Butler, G. C. and Marrian, G. F.: The isolation of 3,17,20-pregnanetriol from the urine of women showing the adrenogenital syndrome. J. Biol. Chem. 1937 **119**: 565.
9. Bongiovanni, A. M.: Unpublished data.
10. Zaffaroni, A.: *Corticosteroid Micro Analysis. Recent Progress in Hormone Research*, Vol. VIII, p. 73, Academic Press Inc., New York, 1953.

SPECTRAL PHONOCARDIOGRAPHY: PROBLEMS AND PROSPECTS IN THE APPLICATION OF THE BELL SOUND SPECTROGRAPH TO PHONOCARDIOGRAPHY*

VICTOR A. McKUSICK, SAMUEL A. TALBOT AND GEORGE N. WEBB

Department of Medicine, The Johns Hopkins University School of Medicine and Hospital

Received for publication February 5, 1954

In the first part of the last decade the Bell Telephone Laboratories developed, under the aegis of Dr. Ralph K. Potter, a method for the visual display of speech sound (1) (2) (3) (4) (5). Inasmuch as the spectrum of frequency content of sound is revealed, the instrument may be called a "sound spectrograph" (2). Intended for such applications as telephony for the deaf and speech training, the type of display obtained has been referred to as "visible speech" (3).

PRINCIPLES OF THE METHOD

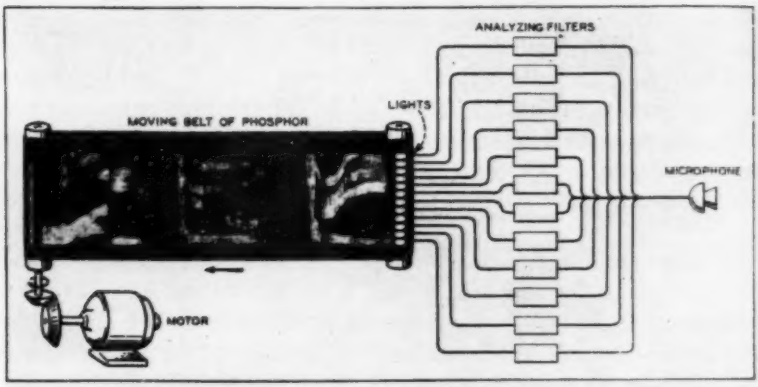
Figure 1, a schematic representation of a direct sound analyzer (4) (5), is useful in understanding the principles of sound spectrography.[1] The whole sound in electrical form passes from the microphone through a battery of filters each of which is tuned to a particular frequency pass-band. For instance, the bottom filter might have a pass-band width of 20–80 c.p.s., the next, 80–140 c.p.s. and so on. Each filter will let through only energy in its particular frequency band. The output from each filter activates a small light behind a rotating phosphorescent belt. The play of lights on this rotating screen provides a pattern which is characteristic of the whole sound applied at the microphone. This display is the sound spectrogram, or in one application "visible speech." Note that the frequency spectrum is displayed on the ordinate, and time on the abscissa. Note further that the intensity of the sounds is represented, at least roughly, by the brightness of a particular area of the screen, inasmuch as the brightness of a given light is a function of the amount of energy in a particular frequency band.

Because of the expense and technical difficulties involved in a direct analyzer to provide high resolution for frequency, an indirect sound spectrograph (2) has been developed along lines indicated schematically in Figure 2. This is in principle the sound spectrograph[2] which we have been using and modifying for

* This work is being supported by a grant from the National Heart Institute of the U.S.P.H.S. and by a contract with the U. S. Air Force (Office of Scientific Research, Air Research and Development Command).

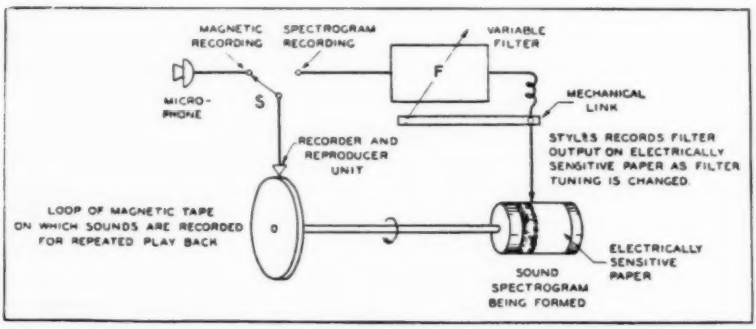
[1] Figures 1, 2, and 3 are reproduced from elsewhere (3) with the permission of the authors and publisher.

[2] Manufactured by the Kay Electric Company, Pine Brook, New Jersey.



Schematic diagram showing how the direct translators convert speech sounds to visible speech patterns.

FIG. 1.


MAGNETIC RECORDING
SPECTROGRAM RECORDING
VARIABLE FILTER
F
MICRO-PHONE
S
MECHANICAL LINK
RECORDER AND REPRODUCER UNIT
STYLUS RECORDS FILTER OUTPUT ON ELECTRICALLY SENSITIVE PAPER AS FILTER TUNING IS CHANGED
LOOP OF MAGNETIC TAPE ON WHICH SOUNDS ARE RECORDED FOR REPEATED PLAY BACK
ELECTRICALLY SENSITIVE PAPER
SOUND SPECTROGRAM BEING FORMED

Schematic diagram of the sound spectrograph illustrating how the analysis of a repeated sample of speech is recorded line by line to form a complete pattern.

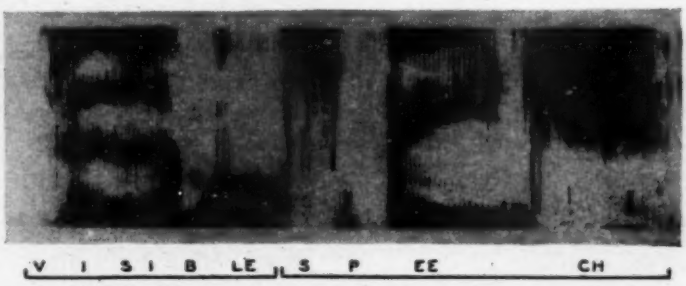
FIG. 2.

phonocardiography. A segment of sound for analysis (6.6 seconds in duration by our present technique) is first recorded on a magnetic disc. This segment is then played back repeatedly through a single analyzing filter. *In effect* the tuning of this filter is changed progressively with each repeated playback of the same segment of sound. The output of the filter marks electrically sensitive paper wrapped on a drum which is rotating on the same axis as the magnetic disc. A "mechanical link" between the marking stylus and the tuning mechanism of the filter provides for synchronization of the progressive change in

effective tuning of the filter and the progressive movement of the stylus up the width of the paper as the drum rotates. Although the same end result is accomplished as by the direct analyzer, several minutes are required for the analysis by the sound spectrograph. (Approximately 100 lines per inch, i.e., 100 revolutions of the drum for each 1 inch of travel of the stylus up the drum, are inscribed regardless of the fundamental pass-band of filter being used or the total frequency span covered in the analysis.) In the case of the sound spectrograph, rather than the entire frequency spectrum being inscribed simultaneously, as in the direct analyzer, the energy content in one frequency band for the entire 6.6 second segment is first recorded, then that for the next band, then the next, and so on. As in the case of the display on the direct translator, *the record from the sound spectrograph has the time dimension as abscissa and the frequency spectrum as ordinate.* In this case, *intensity is indicated roughly by blackness of the mark* made on the electrically sensitive paper by the marking stylus.

In an idealized sense the spectrogram presents to the eye the same information that may be received by ear. The important advantage of picturing the sound is that such displays may be analyzed leisurely, classified like fingerprints, printed in books, compared and discussed in detail. Sound *per se* or in other forms cannot be handled in these ways.

The words "Visible Speech" in the form recorded by the oscillograph.



The words "Visible Speech" as recorded by the sound spectrograph.

Fig. 3. See text.

The advantages of spectral phonocardiography over classical oscillographic phonocardiography and possibly the potentialities of the new method are suggested by the comparison in Figure 3 of an oscillographic recording and a spectrogram of the words "visible speech." The oscillographic recording might be a phonocardiogram of the classical variety; its characteristics are not distinctive. The spectrogram, on the other hand, has a distinctive pattern which permits one to learn, with considerable facility, to read what words are being displayed. Furthermore, one can distinguish a male voice from a female voice and regional accents are represented by distinguishing features in the spectrograms. Application of sound spectrography to phonocardiography was further encouraged by the experience with this method in detecting and identifying defects in complex machines such as airplane motors.

With the sound spectrograph as now used for phonocardiography, the 6.6 second segment of sound is displayed on a time scale 12.5 inches in length. The vertical dimension of the records (frequency scale) is 4 inches and the frequency scale covered in this range is 0 to 750 or 0 to 1500 c.p.s., depending on the requirements of the particular sound being analyzed. If necessary in exceptional instances the scanning can be carried to higher frequencies than 1500 c.p.s. The magnetic recording and reproducing system of the present instrument has a maximum signal-to-noise range of 55 db, although the "sectioner" (frequency × intensity analysis; see below) covers a range of only 35 db.

SPECTRAL PHONOCARDIOGRAMS

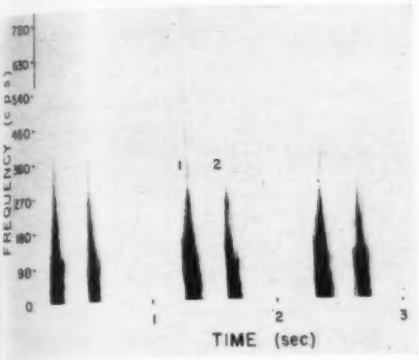
Figure 4A–F presents examples of spectral phonocardiograms.[3] No particular attention has been paid to quantitative (intensity) aspects. In fact, both in the initial recordings and in the playback for analysis the lower frequency range has been attenuated to permit adequate display of the higher frequency components. Although the records indicate content in the higher frequency ranges, the energy in those areas must represent a small part of the total. All these records were made with a pass-band filter width (see below for terms of definition) of 71 c.p.s., the center of the pass-band being shifted 1.75–3.75 c.p.s. with each playback. After appropriate frequency calibration the tracings have been mounted on the frequency × time frames as illustrated.

THE SPECTRAL PHONOCARDIOGRAPH

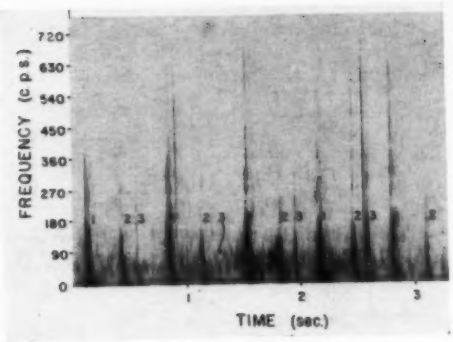
Phonocardiography is one of the most demanding applications of sound spectrography. The following instrumental modifications have been found necessary:

1. Approximately equal resolution for both time and frequency has been at-

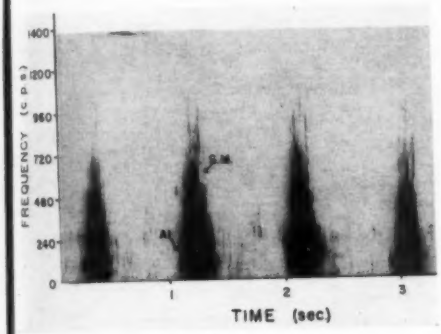
[3] These were made largely from magnetic tape recordings of heart sounds kindly lent by Dr. W. Proctor Harvey of Georgetown University.



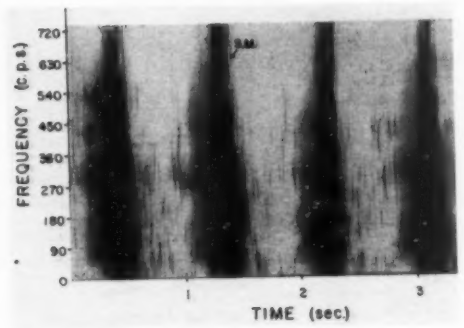
A



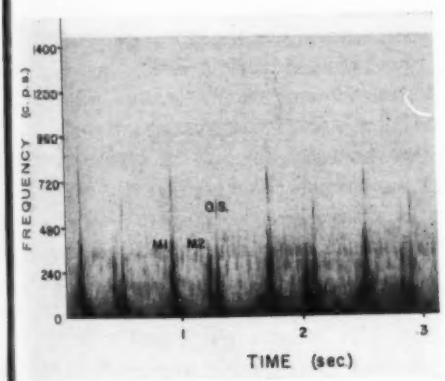
B



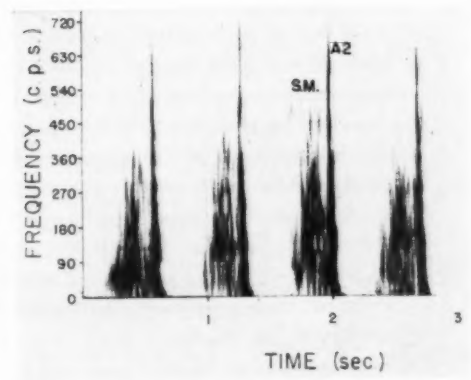
C



D



E



F

FIG. 4. See text for description of the conditions of recording and analyzing. A. Mitral area in a normal subject. B. Normal third heart sound with respiratory variation. (Noncardiac noise is present as well as click artefacts in association with the second S1 and the last S3.) C. Aortic area in aortic stenosis (A2 absent). D. Same as C except different frequency scale. E. Apex in mitral stenosis. Accentuated M1 and opening mitral snap. (Noncardiac background noise is present.) F. Aortic area in hypertension. Systolic murmur and accentuated A2.

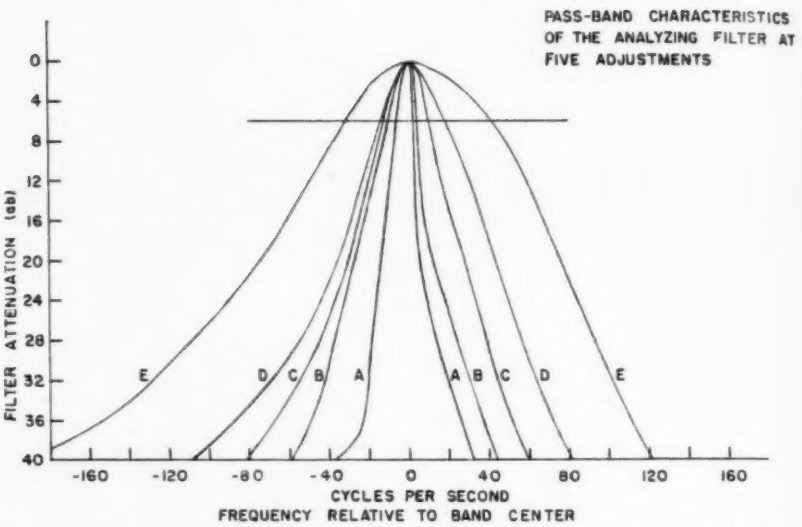


FIG. 5. The pass-band characteristics of five filter set-ups, defined in terms of the width at 6 db attenuation (indicated by the horizontal line).

tained by use of an intermediate width of pass-band. The narrower the frequency pass-band of a filter the more it "rings."[4] "Ringing" results in smudging of the record in the time dimension. Although reasonably high frequency definition in spectral phonocardiography is desirable, the rapid transients of heart sounds must also be followed faithfully. The design of our earlier instrument permitted selection of a pass-band width of 9 c.p.s. or of 71 c.p.s.; of the two the 71 c.p.s. band width produced the more realistic records, i.e., records which look more like the mental image of the sounds (cf. Fig. 4A–F). However, a compromise width of about 23 c.p.s. seems preferable (cf. Fig. 5–7) and may prove satisfactory in general for spectral phonocardiography.

Five filter set-ups, having the characteristics indicated in Figure 5, have been utilized in the testing for optimum width of pass-band. In order, the widths of these pass-bands, indicated by letters A to E, are, at 6 db decrement, roughly 9, 16, 23, 34, and 71 c.p.s., respectively. When a test signal with a repetition rate of 30 per second and harmonics at each 30 c.p.s. is analyzed with each of these pass-bands, the set of records presented in Figure 6 results. The intermittent temporal character of the signal is lost in the 9 c.p.s. analysis whereas the harmonic components are not distinguishable in the 71 c.p.s.

[4] Although perhaps not in the best engineering usage, the word "ringing," referring to the continued oscillations of a filter after the actual signal has ceased, is used here to make this exposition clearer to non-technical readers.

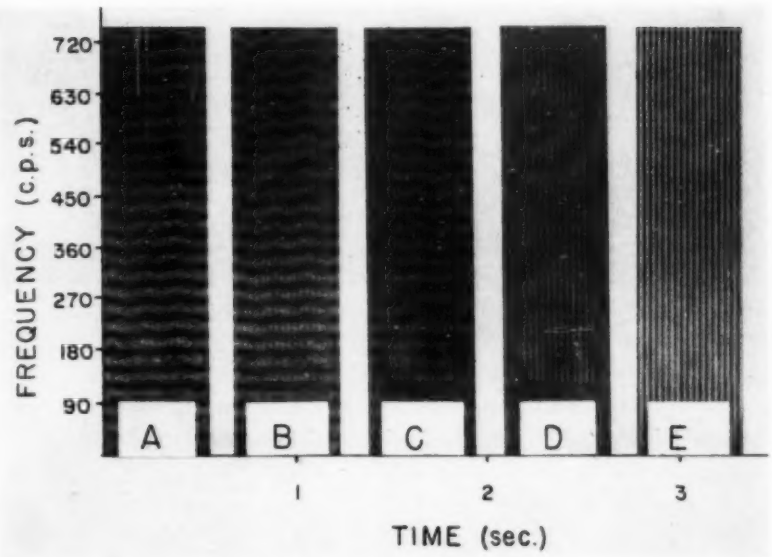


FIG. 6. Analysis, through the five filter set-ups characterized in Figure 5, of a signal with a repetition rate of 30 x/sec. and harmonics at each 30 c.p.s. Note that pass-band C provides best compromise definition of both time and frequency.

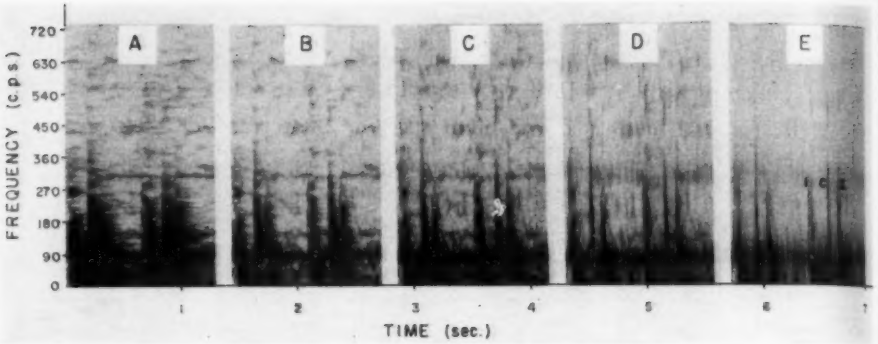
analysis. The 23 c.p.s. filter provides the best compromise. At this pass-band width a grill pattern results in which temporal and harmonic components are displayed with about equal clarity.

Figure 7 a, b, and c presents spectral phonocardiograms recorded by these five filter set-ups. Here, too, the 23 c.p.s. pass-band width appears to provide the best compromise display of both timing and frequency characteristics. Note that "ringing"[4] is greatest in the lower frequency zones where there is greater energy content; the filter is hit harder and "rings" longer.

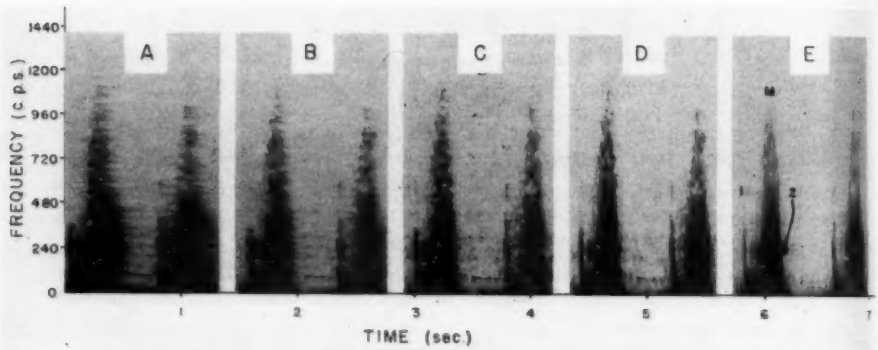
Although the above compromise is a workable method for attaining about equally fine definition for both time and frequency, a method for even higher simultaneous resolution in both parameters should be sought.

2. Display of frequency on a logarithmic scale (i.e., the ordinate) is probably a closer match of the auscultatory experience. At any rate the lower frequency range, where most of the energy content of heart sounds is concentrated, is spread out to advantage.

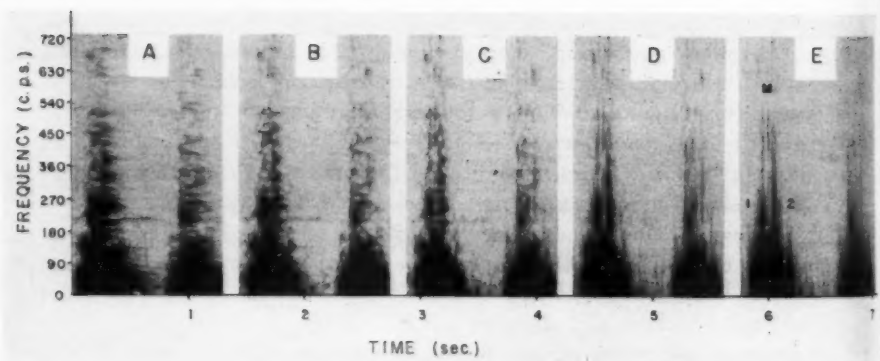
3. Instrumentation for the simultaneous parallel recording of other physiologic data (for timing and correlative purposes) is obviously essential. For this purpose the system which seems to be most practicable is one involving the modulation of a high-frequency carrier band on the same tape on which



A



B



C

FIG. 7. Heart sounds recorded through each of the filter set-ups characterized in Figure 5. Note that pass-band C is the best compromise in each case. A. Systolic click. Note that artefacts such as the band at 90 cycles are readily identified as such. B. Late systolic murmur at the apex. C. Aortic area in aortic stenosis (A2 present).

the basic data are initially recorded (see #5 below). Before the spectral frequency analysis is run through, the electrocardiogram (or other phenomenon as desired) is written in the space at the bottom of the record by means of appropriate pen. For economy of space in the recording of multiple physiologic events, the record of respirations with superimposed "pips" indicating the "R" wave of the EKG and the incisura of the carotid sphygmogram should prove useful.

4. Conversion from the use of direct-writing electrically-sensitive paper (with a visual density range of only 6 db) to photographic film negative (with a range up to 60 db) will improve greatly the definition for intensity. Although the direct-written record may be satisfactory at present for teaching purposes or for inclusion in the patient's unit record the highest possible definition in all three parameters is desirable for investigative purposes.

The commercially available sound spectrograph will also record the same spectrum with much greater intensity detail at preselected points in time (so-called "sectioning"). This process involves, however, an additional time-consuming operation, can be done simultaneously for only a limited number of points (five with the present instrument), and does not satisfy our primary objective—a single record with high resolution in all three dimensions. It is true that serial "sectioning" of heart sounds and murmurs by the existing method, although arduous, is likely to provide useful information. Figure 8 presents "sections" (frequency × intensity displays) at three points in a "cooing" diastolic murmur. Notice that the fundamental and the harmonics are clearly discernible. The intensity scale (linear in Figure 8) may be made logarithmic (i.e., in decibels). The three sets of sections were made with a pass-band of 71, 23, and 9 c.p.s., respectively. The narrower the pass-band the finer the definition of intensity characteristics. As in the case of frequency and time the 23 c.p.s. pass-band provides satisfactory resolution in intensity. Resolution in time, frequency, and intensity is one problem because of the intimate inter-relationship of these three parameters.

5. It is most practical to record the heart sounds first on magnetic tape and then select segments for analysis. This intermediate step increases the flexibility of the method by permitting one to study the heart sounds in dynamic, rapidly changing situations. Each complete analysis on the sound spectrograph takes about 5 minutes. From the tape an oscillographic display of the same segment of sound is made for comparison with the spectrogram. The tape recordings, the raw material of these studies, are preserved for subsequent reference.

Common to all phonocardiography are the problems of selection of microphones, those of chest-microphone coupling, and those of isolation from ambient vibrations, particularly in the lower frequency range. In spectral phonocardiography, too, there are the difficulties of calibration imposed by the

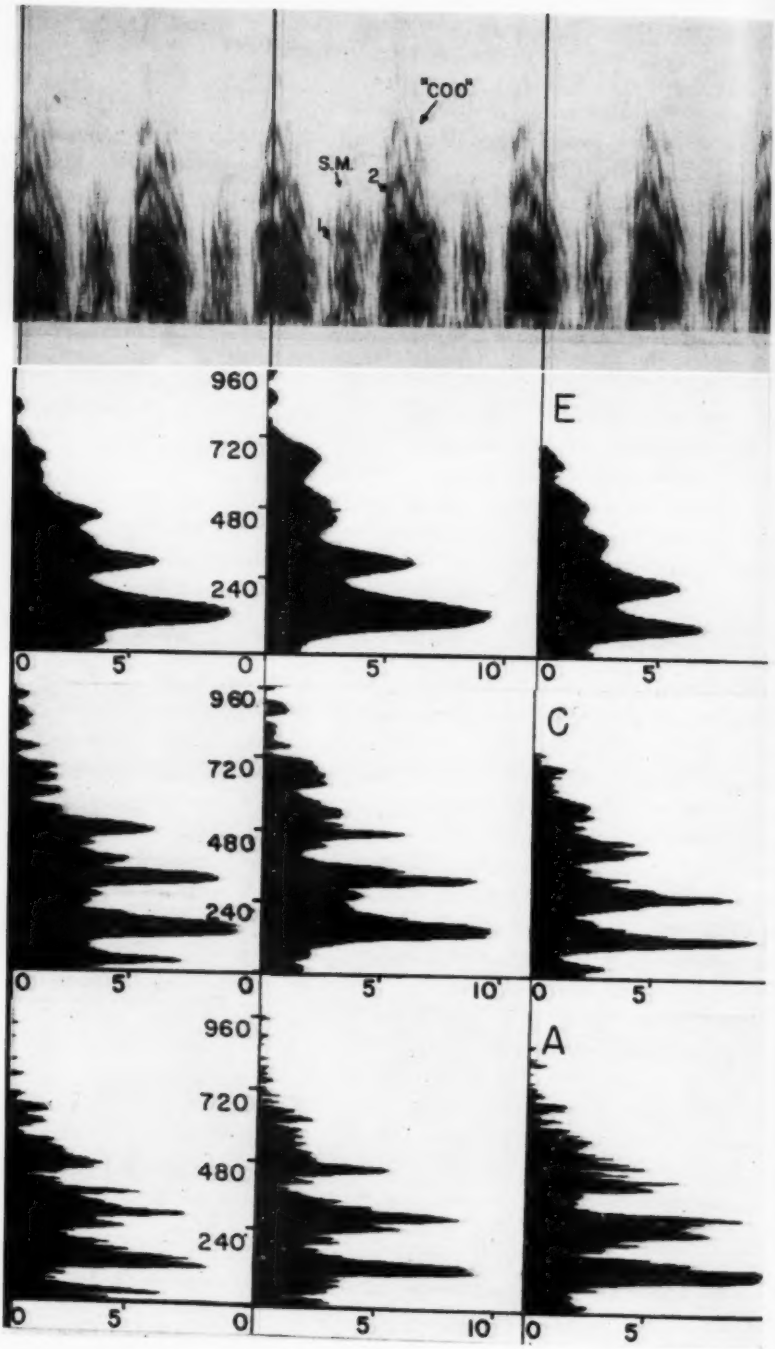


FIG. 8

variable sound-conducting properties of chests. Furthermore, this method shares with tape recording of heart sounds the problem of compressing the great dynamic range (up to 100 db) of heart sounds into the range (approximately 50 db) which the tape will accept.

APPLICATION

For the first time one can expect to be able to incorporate the timing, frequency, and intensity characteristics of heart sounds in a single, objective, detailed, standardizable and analytical display.

We are operating on the basis of the following six hypotheses which are yet to be fully established:

1) Spectral phonocardiograms resemble the mental image of heart sounds and murmurs. Therefore, their interpretation is easy for the experienced stethoscopist and does not require the mastering of an unfamiliar language.

2) For the same reason the pedagogic value of spectral phonocardiograms is considerable. They look not unlike refined versions of the crude chalk diagrams used in teaching heart sounds. Spectral phonocardiographic displays by the direct analyzer method (described briefly above; see Fig. 1) have much to recommend them for use as an integral part of audio-visual devices for the teaching of cardiology.

3) For purposes of clinical record, as, for instance, in the assessment of the progress of disease or of the results of operation, this method has value.

4) Artefacts produced by ambient vibrations—a bugaboo of oscillographic phonocardiography—can, for the most part, be recognized and therefore disregarded in spectral phonocardiography. In this property the new method shares the advantage of the ear.

5) This method of analysis permits the precise physical definition of adjectives (too numerous to list here) used in describing heart sounds. The phonetics of the heart can be studied in detail. (We are concurrently studying the physical basis of the extensive vocabulary used in connection with normal and adventitious respiratory sounds.)

6) Information of physiologic and pathologic significance beyond the appreciation of either the ear or the current methods of phonocardiography is provided. Many problems in connection with clinical auscultation of the heart deserve re-investigation by this method: in particular, the objective differen-

FIG. 8. At the top is the spectral phonocardiogram of a "cooing" aortic diastolic murmur. A systolic murmur is also present. Below are presented frequency x intensity sections at the three specific points in the "coo" indicated by the lines in the spectral phonocardiogram. The frequency pass-band width was 71, 23, and 9 c.p.s., respectively, in the three sets of sections E, C, and A. In each case the frequency scale is the ordinate and intensity (in arbitrary units) the abscissa. See text for further description.

tiation of sounds and murmurs which are of different pathologic significance but of virtually identical stethoscopic character.

Spectral phonocardiography, the designation proposed for this method, has the virtues of retaining the time-honored term "phonocardiography" and of incorporating the concept of spectral, i.e., frequency, display.

ACKNOWLEDGMENT

Dr. Elliot V. Newman guided our initial explorations of spectral phonocardiography. Ralph K. Potter and R. R. Riesz of the Bell Telephone Laboratories have been most helpful.

REFERENCES

1. Potter, R. K.: Introduction to technical discussions of sound portrayal. J. Acoustical Soc. Am. 1946, **18:** 1.
2. Koenig, W., Dunn, H. K. and Lacy, L. Y.: The sound spectrograph. *Ibid* 1946, **18:** 19.
3. Potter, R. K., Kopp, G. A. and Green, H. C.: *Visible Speech.* New York, D. Van Nostrand Co., Inc., 1947.
4. Riesz, R. R. and Schott, L.: Visible speech cathode-ray translator. J. Acoustical Soc. Am. 1946, **18:** 50.
5. Dudley, H. and Gruenz, O. O., Jr.: Visible speech translators with external phosphors. *Ibid* 1946, **18:** 62.

SIMULTANEOUS DYE DILUTION AND FICK DETERMINATIONS OF LEFT AND RIGHT VENTRICULAR OUTPUTS IN DOGS WITH END TO END ANASTOMOSIS BETWEEN THE LEFT SUBCLAVIAN AND LEFT PULMONARY ARTERY

MORTON LEE PEARCE*, HAROLD A. COLLINS, H. WILLIAM SCOTT, JR. AND ELLIOT V. NEWMAN

The Departments of Medicine and Surgery, Vanderbilt University School of Medicine, Nashville, Tennessee

Received for publication February 10, 1954

The simultaneous determination of the separate outputs of the right and left ventricles in the intact animal with centrally located shunts is at best only approximate. The most reliable data have resulted from applications of the direct Fick method, but these have required assumptions as to adequacy of mixing at points of blood sampling (1, 2). It is the purpose of this paper to present a method for these separate determinations and the results obtained by the method in experimentally produced shunts.

Dogs were used in which the left main pulmonary artery was divided and the distal end anastamosed to the proximal end of the divided left subclavian artery (Fig. 1). In these animals the returning venous blood is pumped by the right ventricle through the right lung while the left lung receives arterialized blood from the left ventricle via the subclavian artery. Therefore, the left ventricular output is the sum of blood returning from the right heart via the right lung and the blood shunted via the subclavian artery through the left lung. The flow of blood through the shunt, then, is the left ventricular output minus the right ventricular output.

It has been shown that a lung which receives only arterialized blood does not exchange oxygen to any significant extent (3). Since blood flowing into the left lung in animals prepared with these shunts is arterialized, the calculation of right heart output by the direct Fick principle can be made from determinations of oxygen consumption and right pulmonary arterial and systemic arterial blood oxygen contents. The calculation of left ventricular output using the Stewart-Hamilton dilution principle can be made if systemic arterial dye concentration is determined serially following the injection of dye proximal to the left ventricle.

METHODS

Mongrel dogs weighing 13.7 to 24.1 kgs. were anesthetized with sodium pentobarbital, 30 mg/kg intravenously. A tracheal cannula was inserted and kept tightly in the trachea by

* G. D. Searle Fellow in Experimental Medicine. Present address: Department of Medicine, UCLA Medical School, Los Angeles 24, California. This work was supported in part by the Life Insurance Medical Research Fund.

inflating a balloon cuff at the end of the cannula. The animals breathed spontaneously. After fifteen to twenty minutes of equilibration with the cannula connected to an oxygen-filled Collins respirometer, the main pulmonary artery was catheterized under fluoroscopic control. Immediately after determination of cardiac output, each animal was submitted to thoracotomy. The left pulmonary artery was divided and its proximal end ligated. An anastomosis was performed between the proximal end of the left subclavian artery and the distal end of the left pulmonary artery (Fig. 1). After approximately a week's convalescence the dogs were again anesthetized and simultaneous determinations of cardiac output by the Fick

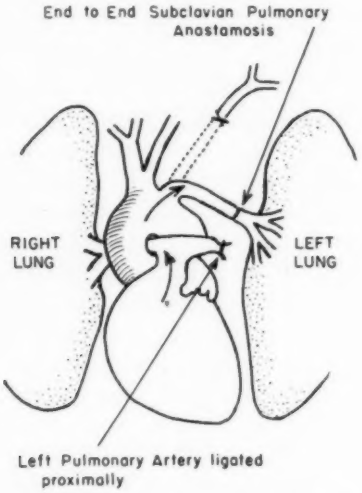


FIG. 1. Diagram of the operative procedure.

and dye methods were made. The Fick determinations were performed as described by Cournand et al. (4), the mixed venous sample being obtained from the main pulmonary artery. The systemic arterial sample was obtained by femoral artery puncture. Oxygen uptake was measured with the respirometer and corrected to standard temperature and pressure. Blood samples were analyzed in duplicate by the Van Slyke manometric method. The cardiac output by the dye method was performed using a photomultiplier densitometer (5) with a carotid curvette (6). Two to four dye curves were recorded simultaneously with the Fick determination. Because an early wave of recirculation markedly altered curves obtained by injecting dye into the right ventricle, the dye was injected through a cardiac catheter into the left auricle or into the left ventricular inflow tract. It has been shown that this technique does not alter the cardiac output values (6).

The validity of these methods for comparative purposes was tested in a series of six dogs without anastomoses. The mean cardiac output by the Fick method was 2.71 l/min. as compared with a mean of 2.86 l/min. by the simultaneous dye method. The standard deviation of the differences was 0.29 l/min.

The surgical methods employed, and long term follow-up studies will be the subject of a separate report (7).

RESULTS AND DISCUSSION

The results are presented in Table I. The cardiac output values are high, presumably because pentobarbital was the anesthetic agent used. However,

TABLE I

Pre-Operative Fick Outputs and Post-Operative Fick and Dye Outputs

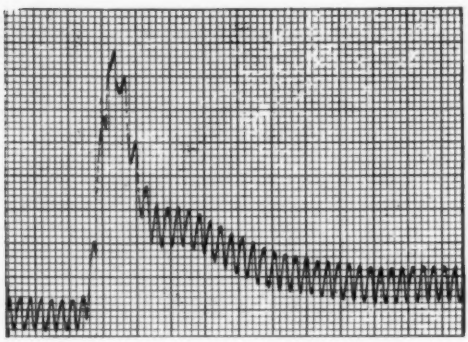
DOG	WEIGHT KG.	PRE-OPERATIVE		POST-OP. DAY	POST-OPERATIVE		
		C.O. (Fick) l./min.	O_2 uptake ml./min.		C.O. (Fick) l./min.	O_2 uptake ml./min.	C.O. (dye) l./min.
1028	24.1	2.95	171	7	7.30	489	10.7
1043	14.2	1.90	78	6	1.06	107	†
43	14.4	2.91	116	7	10.50	488	16.1
50	14.6	4.28	160	7	6.25	278	7.10
56	15.4	3.69	115	8	2.79	191	3.26
64	14.0	6.62	146	9	1.96	157	3.26
68	14.8	8.17	157	9	3.52	151	6.25
77	16.0	5.95	128	5	1.69	151	2.25
78	15.3	3.82	132	5	4.20	256	4.33
89	14.2	4.98	182	10	3.76	147	†
90	14.4	3.22	92	6	1.69	110	4.43
91	13.8	3.92	91	6	1.47	96	6.94
96	13.7	2.26	100	6*	1.52	95	1.67

* Animal expired and shunt found obliterated at autopsy.

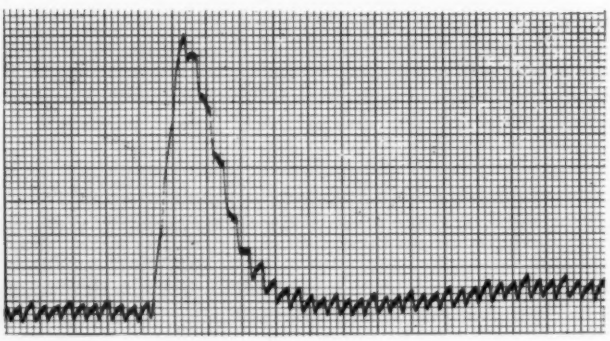
† Technically unsatisfactory determinations.

this does not invalidate the results since each animal was used as his own control and pentobarbital was used throughout. There was a wide variation of cardiac outputs in response to the shunts, the latter ranging from 0.13 to 5.60 l/min. Since the standard deviation of the differences in the two methods is 0.29 l/min., a shunt of at least 0.58 l/min. would have to be present to be significant. This difference existed for most of the determinations. Two of the pre-operative output determinations were high as a result of small A–V differences. This may have been due to an atypical response to the anesthetic agent. Dog 96 died a few hours after the post-operative determination and the anastamosis was found to be blocked by an old organized thrombus. This had been predicted from the comparative Fick and dye cardiac output values of 1.52 l/min. and 1.67 l/min. respectively. Also the absence of a "shoulder" on the descending limb of the dilution curve (Fig. 2b) which is compared with one from an animal with a patent anastomosis (Fig. 2a) had suggested the absence of a functioning left to right shunt. Dogs 56 and 78 showed small shunts. Nevertheless, the dye dilution curves in these animals had "shoulders" on the descending limbs in all ways similar to the other animals. Obviously to explain these discrepancies, further analytical work is needed on the form of dye dilution curves obtained in cases of intrathoracic shunts.

In dogs 1028 and 43 there was a marked rise in right ventricular output following surgery. This was associated more with an elevation of oxygen consumption than with a small A–V oxygen difference. In the entire post-operative group there is a much better correlation between oxygen consumption and right ventricular output than there is in the pre-operative group. The reason for this is



A) Functioning left - right shunt



B) Shunt thrombosed - Animal in failure

FIG. 2. Recordings of arterial time-concentration curves.

not obvious. Possibly the reflex mechanisms controlling cardiac output have been altered by the presence of the shunt.

In most instances there was a rise in oxygen consumption following surgery. This may be due in part to the increased work of the left ventricle, and in part to the increased metabolic demands of wound healing.

SUMMARY

A method is presented for the simultaneous and separate determinations of right and left ventricular outputs in the presence of a left to right shunt. The method was applied to dogs with end to end anastomosis between the left subclavian and left pulmonary arteries.

REFERENCES

(1) EPPINGER, E. C., BURWELL, C. S. AND GROSS, R. E.: The effects of patent ductus arteriosus on the circulation. J. Clin. Inv., 1941, **20:** 127.

(2) Bing, R. V., Vandam, L. D. and Gray, F. D., Jr.: Physiological studies in congenital heart disease. I. Procedures. Bull. Johns Hopkins Hosp., 1947, **80:** 107.

(3) Roh, C. E., Greene, D. G., Himmelstein, A., Humphreys, G. H., III and Baldwin, E. DeF.: Cardiopulmonary function studies in a patient with ligation of the left pulmonary artery. Am. J. Med., 1949, **6:** 795.

(4) Cournand, A., Baldwin, J. S. and Himmelstein, A.: *Cardiac Catheterisation in Congenital Heart Disease.* The Commonwealth Fund, New York, 1949.

(5) Milnor, W. R., Talbot, S. A., McKeever, W. P., Marye, R. B. and Newman, E. V.: A photoelectric ear densitometer for continuously recording the arterial concentration of T-1824 in the dye dilution method. Circulation Research, 1953, **1:** 117.

(6) Pearce, M. L., McKeever, W. P., Dow, P. and Newman, E. V.: The influence of injection site upon the form of dye dilution curves. Circulation Research, 1953, **1:** 112.

(7) Scott, H. W., Jr. et al.: To be published.

STUDIES ON THE DURATION OF PROTECTION AFFORDED BY ACTIVE IMMUNIZATION AGAINST TETANUS[1]

THOMAS B. TURNER, EDWARD S. STAFFORD AND LEON GOLDMAN

The Department of Microbiology and The Department of Surgery of The Johns Hopkins University, and The Surgical Service of The Johns Hopkins Hospital

Received for publication February 24, 1954

Of all the common immunization procedures, active immunization with tetanus toxoid is generally regarded as being among the best from the standpoints of effectiveness, and freedom from untoward reactions; yet its potentialities are scarcely being exploited to the fullest extent, especially in civilian medical practice.

The remarkable record of the anti-tetanus immunization procedure used by the United States during World War II is well known. According to data published by Long and Sartwell (1) there were but 12 cases of tetanus among 2,734,819 hospital admissions for wounds and injuries; of these only 4 had received complete basic immunization and booster injections and of these 4, only *one* died. Thus it can be stated that only one fatal case of tetanus occurred in the U. S. Army (which included the air force at that time) as a result of either battle wound or non-battle injury during World War II among personnel properly immunized.

Nevertheless, certain important questions have been troubling those charged with the responsibility for the treatment of injured civilians since World War II. Chief among these is the status of the protection against tetanus in immunized veterans and in the multitude of children born during the past 15 years, since it has been common practice in most localities to administer tetanus toxoid combined with diphtheria toxoid to infants and school children. Is the protection permanent? If not, at what intervals should booster doses be given? Arbitrary rules of procedure have been adopted in many locales but with little factual basis.

As a consequence, tetanus antitoxin as an emergency measure is being used more frequently than would appear to be necessary or desirable. This situation would occasion little concern were it not for certain undesirable features attendant upon the administration of horse serum. Not only are reactions characteristic of the hypersensitive state, such as serum sickness, fairly common, but severe anaphylactoid reactions either immediate or delayed also occur. Moreover, recent studies by Rich and his associates (2), among others, suggest

[1] This study was supported jointly by a grant to the Department of Microbiology from The Whitehall Foundation, Inc., New York and by research funds from the Department of Surgery.

that *in vivo* antigen-antibody reactions invoked by contact with various sensitizing antigens such as horse serum, sulfonamide drugs, and doubtless many other substances can be accompanied by histological changes which may not be harmless, either in their immediate or in their remote effects. In view of the record of the armed forces referred to above, it seems likely that the actual mortality rate for tetanus among previously immunized personnel may be lower than the mortality rate to be expected from the continued prophylactic use of horse serum tetanus antitoxin in the population at large.

THE STUDY GROUP

The study group comprised 175 persons, all adults, of whom 145 had been previously immunized with tetanus toxoid and 30 had never been so immunized. All persons in the study were associated with The Johns Hopkins Medical Institutions[2] except 23 individuals who were undergoing elective surgery in The Fort Howard Veterans Hospital.[3] Of the 175 persons, 30 were women.

The focal group comprised 72 individuals who had served in the armed forces of the United States or of the British Commonwealth during World War II and whose last previous tetanus toxoid inoculation had been at least 5 years before the time of this study, which extended over the late summer and early fall of 1953. Of these, 41 were physicians, 3 were in one of the allied professions; and 28 were laymen. The history of prior active immunization against tetanus was regarded as unusually accurate in view of the type of individual in the study; in many instances an immunization register or other written record giving precise dates of tetanus toxoid injections was available. All veterans had received a basic series of injections in the armed forces during World War II and nearly all had had at least one booster injection, but none had received toxoid since discharge from the service. In age the members of this group varied widely, from about 28 to over 60 years, but age itself seemed not to influence the pattern of response. In the women included in the study the response to tetanus immunization did not vary significantly from that observed in men and no separate analysis according to sex will be made.

Two other categories were included in the study by way of controls; the one, a group of 73 individuals who had received tetanus toxoid within the past 5 years. Some of these were veterans who had been given a booster dose more recently than 5 years, and some were second year medical students who had received their basic immunization shortly before or upon entrance into medical school. The other control group comprised 30 individuals who neither had served in the armed forces, nor ever received tetanus toxoid.

[2] The authors wish to acknowledge with great appreciation the spirit of cooperation manifested by all who participated in this study at no little inconvenience to themselves.

[3] For the historical data and blood samples on this group we are indebted to Dr. Joseph Miller, Chief of Surgery, Fort Howard Veterans Hospital, and his staff.

METHOD OF STUDY

Blood was collected in the usual manner and the serum separated and placed in the refrigerator until tested. The term "pre-booster specimen" refers to the sample of serum obtained just prior to the administration of a booster dose of toxoid at the time of this study; and post-booster specimens refer to the indicated day after the booster injection.

The booster injection consisted of 0.5 ml. of fluid toxoid[4] injected intramuscularly into the deltoid. Post-booster specimens were taken on the 4th, 7th, and 14th days in most instances.

Titrations for serum antitoxin levels were made in white mice weighing 18 grams ± 2.0 grams. All titrations were made with a single pool of highly purified toxin (Lot No. LCI-10-9-47) prepared and generously supplied to us by Dr. Louis Pillemer of Western Reserve University. This toxin pool contained approximately 4,000,000 mouse MLD's per ml. Aliquots of the pool were maintained at −40°C until used; titrations for potency at the time of each series of tests showed no significant variation during the course of the study. Toxin dilutions were made in beef infusion broth with pH adjusted to 6.6.

The challenge dose of toxin for each mouse consisted of approximately 10 mouse MLD's contained in 0.1 ml. of diluted toxin to which was added 0.1 ml. of the appropriate dilution of serum to be tested. This challenge dose invariably caused death in mice when mixed with serum known to contain no tetanus antitoxin, but when this dose was mixed with an equal volume of Standard Antitoxin,[5] containing 0.001 units, the mice were invariably protected. This amount of antitoxin, however, did not protect against twice the challenge dose employed in the tests. It was assumed, therefore, that the highest dilution of a given serum which in 0.1 ml. amounts protected mice against the challenge dose of toxin contained at least 0.001 units of antitoxin, or 0.01 units per ml. In the early series of tests three mice were customarily inoculated with each serum dilution plus the challenge dose of toxin. Due to the remarkable uniformity of results within a given mouse group the number of mice injected per dilution was subsequently reduced to two. The serum-toxin mixtures in a total volume of 0.2 ml. were injected into the gluteal muscles of the mouse at the base of the tail on the right. Mice were observed for 4 days, death being taken as the end-point. Virtually all mice that died first exhibited muscle paralysis of varying degrees of severity. Mice inoculated with mixtures containing no antitoxin customarily showed paralysis within 24 hours and died within 48 hours.

RESULTS

Tetanus Antitoxin Levels According to Time Elapsed Since Last Toxoid Injection

In Table I are shown the serum antitoxin levels of individuals who had had previous tetanus immunization, distributed according to elapsed time since the last toxoid injection. Of the total persons studied, 72 had received no tetanus toxoid injection for 5–11 years prior to determination of antitoxin levels, while 73 had received at least one injection within the preceding 5 years.

[4] The toxoid used was kindly supplied by Lederle Division, American Cyanamid Company under the designation Tetanus Toxoid Fluid-Purogenated.

[5] The Standard Antitoxin was kindly supplied by Dr. W. G. Workman, Chief of the Biologics Control Division of The National Institutes of Health. The Antitoxin Unit is based on the United States Standard Unit which since 1950 has been equivalent to the Standard International Unit of Tetanus Antitoxin.

TABLE I

Serum Antitoxin Levels According to Time Elapsed Since Last Tetanus Toxoid Injection

YEAR OF LAST TOXOID	YEARS SINCE LAST TOXOID	NUMBER IN GROUP	NUMBER WITH INDICATED SERUM ANTITOXIN LEVEL IN UNITS PER ML.			
			<0.01	0.01	0.1	1.0
42–43	10–11	10	3	3	2	2
44	9	9	1	3	3	2
45	8	27	—	8	15	4
46	7	20	—	5	13	2
47–48	5–6	6	2	2	1	1
Subtotal 42–48	5–11	72	6	21	34	11
49–50	3–4	11	1	—	2	8
51–52	1–2	21	1	1	4	15
51–52	1–2*	26	1	5	12	8
53	<1	15	—	—	1	14
Subtotal 49–53	<5	73	3	6	19	45

* Basic course only—no booster.

In the former group, additional titrations showed that of 21 individuals listed as having 0.01 units of antitoxin per ml., 10 had at least 0.05 units; of 34 listed as having 0.1 units, 13 had at least 0.5 units; of 11 listed as having 1.0 units, none had as much as 5.0 units per ml.

Of the entire group of 145 only 9 or 6.2 per cent had levels below 0.01 units. The median antitoxin level was 10-fold lower in the 5–11 year group than in those who had had a more recent booster dose, but even in the former group 75 per cent had serum antitoxin levels of 0.5 units or higher. Of those who had had an immunizing injection within 5 years, 93 per cent had levels of 0.05 units or higher.

It should be noted that the higher titers were observed in those individuals who had received repeated booster injections of tetanus toxoid. In the 5–11 year group only 5 individuals had received only the basic course of 2 or 3 immunizing injections. The titers in these individuals and the year of basic immunization were as follows: <0.01 units, 1942; 1.0 units, 1943; 0.01 units, 1943; 0.05 units, 1945; <0.01 units, 1948.

Similarly, in the group of medical students who had received a basic course of injections one to two years previously but no booster injections of toxoid, the serum antitoxin levels tended to be lower than in those who had received at least one booster injection.

The foregoing results are in line with those reported by others, although in such reports only fragmentary data are available for persons who have had

no immunizing injections for 5 years or longer. Numerous studies have been reported in which serum antitoxin levels have been determined after 3–5 years (1, 3–6) and some of these reports contain data on a few individuals who had not received stimulating doses of toxoid for longer periods of time.

Those studies as well as our own indicate that individuals who have had the basic course of immunization only, without subsequent booster doses, show significantly lower serum antitoxin levels on the whole, than do persons who have had one or more booster doses of toxoid. In our series for example were a number of regular officers of the armed services who maintain very high levels, apparently as a result of repeated stimulating doses.

Speed and Degree of Antitoxin Response to Booster Doses of Toxoid

It has been shown by a number of investigators (6, 7) that individuals recently immunized with tetanus toxoid respond with a detectable rise in antitoxin levels within 4 to 7 days following a booster dose of toxoid. It will be evident from the data shown in Tables II and III that such a rise occurs with great uniformity in individuals who have had no immunizing injections for

TABLE II

Serum Antitoxin Levels 4 Days After Booster Injection of Tetanus Toxoid, Among Individuals Whose Last Previous Immunizing Injection Was 5-11 Years Earlier

Pre-booster Antitoxin Level	Number in Group	Number Showing Indicated Level in Units Per Ml.						
		<0.01	0.01	0.05	0.1	0.5	1.0	5.0
<0.01	3	2	1					
0.01	8		4	3	1			
0.05	7			4	1	2	-	-
0.1	12				8	1	3	-
0.5	11				2	6	3	-
1.0	7						7	-

TABLE III

Serum Antitoxin Levels 7 Days After Booster Injection of Tetanus Toxoid, Among Individuals Whose Last Previous Immunizing Injection Was 5-11 Years Earlier

Pre-booster Antitoxin Level	Number in Group	Number Showing Indicated Level in Units Per Ml.							
		<0.01	0.01	0.05	0.1	0.5	1.0	5.0	10.0
<0.01	4	-	2	-	-	1	-	1	-
0.01	8		-	-	-		4	2	2
0.05	9					2	3	-	4
0.1	15						2	3	10
0.5	10						1	1	8
1.0	8								8

5–11 years before the test dose. Furthermore, this rapid anamnestic response was demonstrable regardless of the pre-booster serum antitoxin level.

For example, within a period of 4 days, in excess of one-third of those tested had a demonstrable rise when the pre-booster titer was less than 1.0 unit per ml. Within 7 days antitoxin rises were demonstrable in all of 53 individuals, with the increase in titer being of the order of 100-fold or higher in most, particularly in those with the lower initial titers. The high levels reached by the 7th day suggest that the rise probably begins in the majority of such individuals about the 4th or 5th day.

Additional determinations of antitoxin levels were not made routinely on persons who showed 5.0 units or more on the 7th day. However, of 15 individuals who showed less than 5.0 units of antitoxin on the 7th day, tests were made on 14-day specimens in 10; 8 of these showed 5.0 units or more, while in 2 the 14 day level was 1.0 units per ml.

Among individuals who had less than 0.01 units before the booster injections, the poorest booster response (to 0.01 units in 7 days) was noted in two persons; the one had received a basic course in 1942 while serving in the armed forces with the last booster dose being in 1943; the other, a medical student had received only a basic course in 1948. Five others with pre-booster titers of less than 0.01 units showed rises to 0.25 units or higher in 7 days.

In addition to the results of booster injections shown in Table III, titrations were made in 8 persons whose last previous immunizing injection was within 5 years. In every instance substantial rises in serum antitoxin were noted on the 7th day, the general pattern conforming to that shown in Table III.

In relatively few other studies, however, have observations been made on the speed of the rise in serum antitoxin levels during the days immediately following the booster dose, in individuals who had had no stimulating doses for 5 years or longer. Bigler (6) studied three children, the elapsed time between the last previous immunizing injection and the booster dose being 7 years 11 months, 4 years 3 months, and 6 months, respectively. Daily serum antitoxin levels were determined after a booster dose of 0.5 ml. of fluid toxoid. In two (the first and third mentioned above) there was a detectable rise on the *third* day and in the other on the 4th day. In one of our cases, too, a test made on the 3rd day instead of the 4th as planned, showed a detectable rise.

Baird (5) in a series of 21 persons given a booster injection 4 to 6 years after the last previous immunization found a substantial rise in serum antitoxin levels in all by the 5th day; 7 of these individuals had had no booster dose for 5 years and 1 for 6 years.

In the studies reported by Peshkin (3) and McBryde and Poston (4) there were altogether 40 children who received a booster injection 4 to 5 years after the last previous toxoid and all showed a rise in serum antitoxin level at 7 days.

Miller, Ryan and Beard (7) studying the question of the relationship of the

type of primary immunization and the type of secondary stimulation to the speed of the antitoxin response, found that children in whom the basic immunization had been with alum precipitated toxoid responded more rapidly than those in whom the primary immunization had been with fluid toxoid. The speed of response however was more rapid when the stimulating dose was fluid tetanus toxoid. For example, among children whose primary immunization had been with alum precipitated toxoid the secondary response to a booster dose of fluid toxoid was as follows: number showing serum antitoxin rises on 4th day, 13 of 18; on the 5th day 27 of 27. While among those receiving alum precipitated toxoid as the booster dose 12 of 31 showed rises on the 4th day, and 26 of 34 on the 5th day. Only a few children in that series had an interval as long as 5 years between primary immunization and booster injection.

Serum Antitoxin Levels After One Injection of Toxoid in Previously Un-immunized Persons

Of 30 individuals who gave no history of previous tetanus immunization, none showed detectable levels of tetanus antitoxin in their serum. Fifteen of these persons were given one injection of 0.5 ml. of fluid tetanus toxoid. There was no demonstrable antitoxin in the serum of 10 after 7 days, nor in 15 after 14 days. Twenty-eight day specimens were taken on 4 persons all of whom showed no evidence of antitoxin on the routine test.

Sera of three persons were tested with one-fifth the standard dose of toxin (i.e., approximately 2 MLD's); none of the sera taken before immunization nor those taken at 7 and 14 days post-immunization showed detectable levels of antitoxin, but 1 of 3 sera taken on the 28th day did protect the injected mice. These results conform in general to results obtained by others when fluid toxoid is used as the immunizing material.

Serum Antitoxin Levels Following Passive Immunization

Frequently overlooked is the fact that following intramuscular administration the maximum concentration of tetanus antitoxin in the blood is often not reached until the second or third day, and that serum antitoxin levels might be quite low during the first 24 hours. Moreover, in patients who develop serum sickness or anaphylactoid reactions, elimination of antitoxin occurs more rapidly than usual.

In the series of patients studied by Gold and Bachers (8) the average serum antitoxin level following intramuscular injection of 1500 units was 0.1 units at 24 hours, 0.25 units at 4 days and 0.15 units at 7 days. In Sneath's series (9) the values were somewhat lower, being 0.1 units at 24 hours, 0.15 units at 3 days, 0.12 units at 5 days, and 0.1 units at 7 days.

Limited observations on antitoxin levels following passive immunization were made during the course of the present study, with results which are in

TABLE IV

Serum Antitoxin Levels Following Administration of 1500 Units of Tetanus Antitoxin

PATIENT	TIME AFTER ANTITOXIN ADMINISTRATION	SERUM ANTITOXIN LEVEL—UNITS/ML.
A. F.	8 hrs.	0.01
J. W.	9 hrs.	0.15
H. C.	11 hrs.	0.05
H. C.	6 days	0.15
W. D.*	14 hrs.	0.25
J. J.	19 hrs.	0.05
A. S.	22 hrs.	0.1
J. T.†	34 hrs.	0.15
J. T.	60 hrs.	0.10
J. D.	44 hrs.	0.25
R. A.‡	immediately before	0.1
	11 hrs.	0.4
	36 hrs.	0.6
	4 days	0.6
	6 days	0.4
	8 days	0.4
	11 days	0.4

* Age 10 years.

† Severe anaphylactoid reaction 8 hrs. after injection of antitoxin.

‡ Patient received three blood transfusions during first 24 hours.

line with those just cited. These data are shown in Table IV. All patients except one (W. D.) were adults; all received one intramuscular injection of 1500 units of tetanus antitoxin in horse serum.

One patient (J. T.) developed a severe anaphylactoid reaction about eight hours after administration of antitoxin and was admitted to the wards in a state of shock. Serum levels in this individual at 34 and 60 hours suggest that antitoxin was being eliminated at an accelerated rate.

On the other hand, if tetanus antitoxin is given in an homologous serum as by transfusion or through plasma injections, high levels may be reached promptly and maintained for longer periods of time. For example, one of our patients (R. A.) was given the usual dose of 1500 units of antitoxin, but no toxoid. A serum specimen taken before administration of antitoxin showed 0.1 units of antitoxin per ml. indicating that the patient had been previously actively immunized. Because of the severity of his injuries three blood transfusions were given prior to serum antitoxin determinations made 11 hours after admission to the hospital, and another transfusion was given on the second day. It will be noted in Table IV that serum antitoxin levels reached 0.4 units within 11 hours and remained at this level or higher for 11 days. While unfortunately no test for tetanus antitoxin was made on the transfused blood, it seems evident that one or more of the 500 ml. amounts given was

obtained from an actively immunized persons who had a fairly high level of antitoxin.

DISCUSSION

Since the development and introduction of tetanus toxoid as an immunizing agent by Ramon and his associates in 1925 (10) the basic immunologic pattern has been fairly well delineated. In a previously unimmunized individual one dose of fluid toxoid or alum precipitated toxoid gives rise to barely detectable levels of antitoxin. A second injection, particularly if an optimum period of 4–6 weeks has elapsed since the first injection, will evoke substantially higher levels of serum antitoxin, and a third dose similarly spaced gives rise to even higher levels which persist for a period of years. Two doses of alum precipitated toxoid seem to accomplish about the same as three injections of a fluid toxoid, in terms of antitoxin production (7, 11).

Following the basic immunizing course, there is a tendency for serum antitoxin levels to decline with time; in general those persons who receive one or more booster doses some months or years after the primary stimulus maintain higher levels for longer periods than do those who have had no secondary stimulating injections. It seems clear too that there are considerable individual variations, not only in the initial response, but in the maintenance of antitoxin levels.

What is a Protective Level of Antitoxin?

While therefore the general pattern of serum antitoxin levels is clear, certain fundamental questions remain unanswered. Among the most important of these is the relationship of antitoxin levels to actual protection against clinical tetanus and death. There are considerable data obtained from experimental animals in the older literature showing the relationship between the size of the dose of antitoxin and protection against toxin injections, but blood levels were not usually determined in these studies. A significant fact usually overlooked is that from shortly after the beginning of World War I until just before the end of that war the standard initial dose of antitoxin in the British Army was 500 units (12). In June, 1917 the routine procedure was modified to include the further administration of 500 units at weekly intervals for 3 doses, since McConkey and Homer (13) had shown that while comparable doses in guinea pigs afforded adequate protection for a week, this protection began to wane about the 7th day; indeed, on a dosage-weight basis the latter authors showed that the equivalent of 220 units afforded good protection for a week.

In the closing months of the war the initial dose was changed to 1500 units, but this was done in the hope of prolonging the effect of antitoxin and not with the idea that the initial dose was too small. Nevertheless, 1500 units of antitoxin has remained the usual prophylactic dose administered to injured or

wounded patients. Since this amount produces in adults a serum antitoxin level of 0.1 to 0.2 units, the inference has been drawn that this level of antitoxin is protective. It is not at all certain, however, that much lower levels of antitoxin may not afford substantial degrees of protection, and indeed the experience in the British Army in World War I suggests that in the order of one-third of this amount (i.e., 0.03–0.06 units) may be adequate under most circumstances.

Moreover, in persons in whom the cells of the body have participated in the immune process through active immunization, it is not known whether serum antitoxin levels constitute a reliable guide to the degree of protection enjoyed by the individual. Phair (14) in attempting to correlate the cutaneous Schick reaction in human beings with the level of diphtheria antitoxin in the blood serum, found that nearly one-fourth of the individuals in one study exhibited a negative Schick test (indicative of immunity) even though no antitoxin or at best a very small amount could be demonstrated in the blood serum. Since rises in serum antitoxin could be detected in some persons by the third day after injection of the small amount of toxin employed in the Schick test, the author suggests that sufficient amounts of antitoxin may be mobilized locally to neutralize the injected toxin before damage to cutaneous tissue could be produced.

The results of the present study on tetanus, as well as data obtained by others, must be viewed in the light of these considerations. It is the authors' opinion, as yet unsupported by adequate clinical or experimental evidence, that regardless of the level of serum antitoxin, any individual who has been actively immunized enjoys a substantial degree of protection against the development of clinical tetanus. The rarity of reports of tetanus occurring in previously immunized persons tends to support this opinion.

The Immune Status of Population Groups

From the data presented in this study together with those already available in the literature it seems clear that in this country there are at least two large segments of the population the members of which not only carry detectable levels of serum antitoxin but who also have the capacity to respond rapidly to a stimulating dose of toxoid. First, there is the huge segment of the adult population which has served in the Armed Forces either during or subsequent to World War II. This is an easily identifiable group from the standpoint of tetanus immunity.

Less easy to delineate but nonetheless important quantitatively are the vast number of children who have received tetanus immunization during the past 15 years. Unfortunately, immunization practice has varied widely with reference to this group. Those who have come under the care of pediatricians in private practice have in general been thoroughly immunized. Moreover, in many health jurisdictions immunization programs directed to the pre-school

and the school child include tetanus toxoid, and such programs each year reach larger numbers of children.

There seems little doubt therefore that through these circumstances the country enjoys a health asset of the first magnitude. How may this asset be preserved, extended, and utilized to the best advantage?

Maintenance of Tetanus Immunity

With such a large proportion of the general population exhibiting good basic immunity to tetanus a concerted effort should be made to preserve this state through booster inoculations of toxoid at more or less regular intervals. What this interval should be is debatable. One injection of either fluid or alum precipitated toxoid every 5 years would seem adequate to maintain a high order of immunity, while in most individuals a booster dose every 10 years would probably afford good protection.

It is greatly to be desired therefore that private practitioners, industrial physicians, and private and public health services initiate a program looking toward the maintenance of a high level of tetanus immunity in the general population. It is scarcely necessary to point out that such a program is consonant with the needs of civil defense.

Management of Injuries in the Civilian Population from the Standpoint of Tetanus Prevention

In an individual previously immunized against tetanus a booster dose of toxoid at the time of injury will, with few exceptions, produce by the 4th or 5th day a level of antitoxin which is generally regarded as protective. Practically, therefore, the problem is one which concerns the first four days after injury. Unfortunately, there are serious gaps in our knowledge of the host-parasite interaction during this critical period.

It is probable that massive contamination with tetanus organisms or spores does not occur nearly so frequently among civilian injured as among military, particularly under certain types of field operations. Moreover, with modern surgical procedures and the use of antibiotics, conditions favorable to the growth of the tetanus organisms probably occur less frequently than formerly.

These two factors together would tend to cause tetanus toxin to be elaborated slowly, if at all, thus giving opportunity not only for neutralization of the toxin, but for the further buildup of serum levels through the booster effect of the natural infection. In an individual with only 0.01 units of antitoxin per ml. of serum, the total antitoxin content of the blood, not to mention the cellular and intracellular fluid, is in the order of 25 units (roughly $\frac{5500}{2}$ ml. blood $\times$ 0.01).

The report by Jones and Jamieson (15) is of interest in this connection. Immunized guinea pigs when given tetanus spores along with calcium chloride (which uniformly caused fatal tetanus in unimmunized pigs) failed to develop tetanus and showed no increase in antitoxin level. This suggests that active immunity may have an antibacterial as well as an anti-toxic action.

Consideration of these hypotheses in relation to the infrequency of clinical tetanus in previously actively immunized individuals and the known toxicity of horse serum, provides justification for the use of toxoid alone (no antitoxin) in any individual who has been previously actively immunized. It is assumed, of course, that the local treatment at the site of injury will follow accepted surgical practice with reference to débridement and the administration of antibiotics where indicated.

Persons who have not had previous active immunization cannot be expected to develop adequate protection from the administration of toxoid within the usual incubation period of tetanus, and for these individuals the administration of antitoxin upon injury is essential. In such instances, tetanus toxoid should always be given simultaneously, but at a different site, with tetanus antitoxin. It has been clearly shown by Miller *et al.* (7) and Gold and Bachers (8) that simultaneous administration does not inhibit the biologic action of either product. If therefore the injured person has been previously immunized additional protection will be afforded, while if he has not, the administration of toxoid will lay the basis for subsequent active immunity. Needless to say, the patient should be instructed with reference to further doses of tetanus toxoid.

Human Serum as a Source of Tetanus Antitoxin

The dangers inherent in the use of horse serum in the prevention and treatment of tetanus in man cannot be over-emphasized. Indeed, some clinicians believe that some of the deaths attributed to tetanus are in fact due to the administration of large amounts of tetanus antitoxin in horse serum, particularly when given by the intravenous route (16). Mortality data in this disease may therefore have to be re-evaluated in relation to this factor.

Consideration should be given to the feasibility of using human serum as a vehicle for tetanus antitoxin in the treatment and possibly in the prevention of tetanus. Such homologous antibody could be administered either through transfusion of whole blood or plasma or as gamma globulin.

Serum antitoxin levels of 20.0 units per ml. are not difficult to produce in man. The usual bleeding of 500 ml. would in such a case contain approximately 5000 units of tetanus antitoxin $\left(\frac{500}{2} \times 20\right)$; approximately the same amount would be contained in 7.0 ml. of gamma globulin prepared from the serum of individuals with this level of antitoxin. Preparation of special gamma globulin solutions with high tetanus antitoxin content would appear to be entirely

feasible, and the use of such preparation in the treatment of patients with tetanus would have many obvious advantages.

Moreover, if the day comes when a large proportion of the general population is actively immunized against tetanus, so that the prophylactic use of antitoxin can be limited to the relatively few individuals who have not been actively immunized, it might be entirely practicable to obtain and process enough human serum in the form of gamma globulin, packaged to contain in the order of 1500 units of tetanus antitoxin, to supply all requirements.

SUMMARY AND CONCLUSIONS

1. Serum antitoxin determinations were made on 145 adults previously immunized with tetanus toxoid. Of these, 72 had received no immunizing injection of tetanus toxoid for 5–11 years, while 73 had received such injections more recently.

2. Among the former group serum antitoxin levels were as follows: less than 0.01 units per ml., 6; 0.01 units, 21; 0.1 units, 34; 1.0 units or greater, 11. Among those with more recent booster doses levels were as follows: less than 0.01 units, 3; 0.01 units, 6; 0.1 units, 19; 1.0 units or greater, 45.

3. Upon administration of a stimulating dose of 0.5 ml. fluid tetanus toxoid to the former group, of 48 individuals tested 4 days later, approximately one-third showed significant rises in serum antitoxin titer; all of 54 tested on the 7th day showed from 10-fold to 1000-fold rises in titer. Among those with more recent immunizing doses, the response to booster injection followed the same pattern.

4. After reviewing the pertinent data on antitoxin levels following passive immunization, and considering such facts as the undesirable effects of repeated use of horse serum, the rarity of clinical tetanus in previously immunized persons, and the results obtained in this study, it is recommended that in civilian medical practice tetanus toxoid alone be administered when needed in the treatment of injuries sustained by individuals known to have had active service in the armed forces of the United States during or subsequent to World War II. The same practice in other individuals, both children and adults, is probably safe when there is a reliable history of previous active tetanus immunization.

5. Once basic tetanus immunization has been established, stimulating doses at 5-year intervals are recommended, although the evidence suggests that booster doses even at 10-year intervals would provide satisfactory antitoxin levels in the great majority of persons.

6. Efforts should continuously be made to enlarge the proportion of our population enjoying basic immunity to tetanus. As one aspect of this campaign, toxoid should always be administered simultaneously, but at a different site, with tetanus antitoxin. A second reason for this step is the added protection

afforded the individual who has been previously immunized but cannot or does not give such a history.

7. In view of the high levels of serum antitoxin attained in many actively immunized persons, consideration should be given to the utilization of such human serum in the treatment of tetanus, and ultimately in its prophylaxis, because of the frequency and severity of reactions from antitoxin given in horse serum.

BIBLIOGRAPHY

1. Long, A. P. and Sartwell, P. E.: Tetanus in the United States Army in World War II. Bull. U. S. Army Med. Dept., 1947, **7:** 371.
2. Rich, A. R.: Hypersensitivity in disease. The Harvey Lectures, 1946–1947, **42:** 106.
3. Peshkin, M. M.: Immunity to tetanus induced by a third dose of toxoid four years after basic immunization. Am. J. Dis. Child., 1945, **69:** 83.
4. McBryde, A. and Poston, M. A.: Immunization with tetanus toxoid. The persistence of antitoxin and the effect of stimulating doses of alum precipitated tetanus toxoid after a five year period. J. Pediat., 1946, **28:** 692.
5. Baird, H. W.: The duration of immunity to tetanus. Yale J. Biol. and Med., 1948–1949, **21:** 385.
6. Bigler, J. A.: Tetanus immunization. Am. J. Dis. Child., 1951, **81:** 226.
7. Miller, J. J., Jr., Ryan, M. L. and Beard, R. R.: The speed of the secondary immune response to tetanus toxoid with a review of war reports and observations on simultaneous injection of toxoid and antitoxin. Pediatrics, 1949, **3:** 64.
8. Gold, H. and Bachers, H.: Combined active-passive immunization against tetanus. J. Immunol., 1943, **47:** 335.
9. Sneath, P. A. T.: The degree of protection provided by tetanus toxoid. Canad. Pub. Health J., 1934, **25:** 195.
10. Ramon, G. and Laffaille, A.: Immunization against tetanus. Compt. rend. Soc. biol., 1925, **93:** 582.
11. Edsall, G.: Immunization of adults against diphtheria and tetanus. Am. J. Pub. Health, 1952, **42:** 393.
12. *History of the Great War: Based on Official Documents. Medical Services (Surgery) of the War.* Edited by Maj. Gen. Sir W. G. Macpherson. Vol. I, Chapter VII (Tetanus) p. 151. Published and printed by His Majesty's Stationery Office, London, England, 1922.
13. McConkey, A. T. and Homer, A.: On the passive immunity conferred by a prophylactic dose of antitetanic serum. Lancet, 1917, **1:** 259.
14. Phair, J. J.: Diphtheria immunization: The interpretation of the Schick test. Am. J. Hyg., 1942, **36:** 283.
15. Jones, F. G. and Jamieson, W. A.: Studies on tetanus toxoid. III. Antitoxin response in guinea pigs immunized with alum precipitated toxoid followed by tetanus spores. J. Bact., 1936, **32:** 33.
16. Dietrich, H. F.: Tetanus in childhood, with special reference to treatment. Am. J. Dis. Child., 1940, **59:** 693.

ABSTRACTS OF PAPERS PRESENTED BEFORE THE JOHNS HOPKINS MEDICAL SOCIETY

February 8, 1954

NEUROPHYSIOLOGICAL STUDY OF A VIRUS DISEASE

(pseudorabies virus in rat sympathetic ganglia)

JOHN DEMPSHER AND MARTIN G. LARRABEE

Pseudorabies virus injected intraocularly into rats infects the ipsilateral superior cervical ganglion and causes spontaneous, intermittent discharges of nerve impulses over the preganglionic and postganglionic nerves. This abnormal activity was studied in situ as well as after excision. In both instances the activity was picked up by platinum electrodes, amplified by capacity-coupled amplifiers, and fed into a double beam cathode ray tube. Several stages of the ganglionic infection have been identified by studying ganglia at different stages of the disease. Impulses began to be discharged over the postganglionic nerve two or three days after inoculation; discharge over the preganglionic nerve began somewhat later; finally, the postganglionic discharge disappeared along with the capacity for synaptic transmission through the ganglion, while the preganglionic activity continued. At the time functional changes began, there was already present a considerable amount of virus, as well as extensive damage to nerve cells. The cellular changes consisted of nuclear inclusion bodies and chromatolysis of Nissl substance.

CLINICAL ASPECTS OF GENETIC VARIANTS OF SICKLEMIA

ERNEST W. SMITH AND C. LOCKARD CONLEY

By means of genetic studies together with electrophoresis of the hemoglobin and other hematologic examinations, twenty-two individuals who were heterozygous for sickling were found to have inherited an additional red cell abnormality. Sixteen patients were found to have sickle cell-hemoglobin C disease, three to have sickle cell-thalassemia disease, two remain unclassified, and one had sickle cell-congenital hemolytic jaundice. The clinical and hematologic manifestations displayed by these patients were compared with the manifestations of classical sickle cell anemia. In every case the genetic variants followed a clinical course which was atypical with respect to sickle cell anemia.

With but two exceptions patients with sickle cell anemia had an unremitting hemolytic anemia, all developed evidences of the disease during the first ten years of life, and none was found to have lived past age forty. None of the patients with genetic variants had constant anemia and several were never found to be anemic. Disease symptoms developed during childhood in less than one-half of these patients, and one remained asymptomatic at age 63. The oldest patient of the group who died at age seventy-five had sickle cell-hemoglobin C disease. Typical sickle cell crises rarely occurred in sickle cell-hemoglobin C disease but were more frequent in sickle cell-thalassemia disease. Only one patient with sickle cell-hemoglobin C disease and none with sickle cell-thalassemia disease had the sickle cell habitus. Leg ulcers were present only in the patient with sickle cell-congenital hemolytic jaundice. Tortuous retinal veins were seen in only three patients of the variant groups. Cardiac abnormalities were rarely encountered in these patients and were invariably of minor significance.

Certain hematologic and clinical features uncommon to sickle cell anemia were frequently encountered in the study of patients with sicklemia variants. Many target cells were seen in the blood smears from all of these patients and particularly striking was the presence of greater than 50 per cent target forms in every patient with sickle cell-hemoglobin C disease. Splenomegaly, not encountered in any patient with sickle cell anemia after age ten, was present in ten of the sixteen patients with sickle cell-hemoglobin C disease, one of the three with sickle cell-thalassemia disease, both of the unclassified patients, and the patient with sickle cell-congenital hemolytic jaundice. Gross, unilateral, renal hematuria occurred in three patients with sickle cell-hemoglobin C disease and in two constituted the major clinical problem over a period of years. Severe crises associated with pregnancy had not occurred in a group of patients with sickle cell anemia, but had occurred in the five patients of the sickle cell-hemoglobin C group who had borne children. Three of these patients had been previously asymptomatic and a fourth had been clinically well since early childhood. Only three maternal deaths associated with sicklemia occurred in the Johns Hopkins Hospital over a twenty-five year period. All three were patients with sickle cell-hemoglobin C disease. Aseptic necrosis of the femoral or humeral heads has not, to our knowledge, occurred in a patient proved to have sickle cell anemia, but was present in five of the sixteen patients with sickle cell-hemoglobin C disease, and has been reported to occur in sickle cell-thalassemia disease.

The patient with sickle cell-congenital hemolytic jaundice had been considered to have sickle cell anemia for many years, but following splenectomy there was complete remission of the hemolytic anemia, jaundice, and leg ulcers. Sickling and spherocytes, however, remained.

ROTATIONAL ROENTGEN THERAPY FOR CARCINOMA OF THE ESOPHAGUS. A CASE REPORT

LOWELL S. MILLER

C. H. (Johns Hopkins History number 644690), a 59 year old negro male, was found to have undifferentiated carcinoma of the thoracic esophagus. Rotational roentgen therapy was given (tumor dose 6540r/35 days). Five and one-half months later pathologic study of the resected esophagus revealed mucosal ulceration but no evidence of tumor.

SOME EFFECTS OF LONG CONTINUED ADMINISTRATION OF STILBESTROL ON THE KIDNEY AND REPRODUCTIVE ORGANS OF THE HAMSTER

ROBERT L. BACON

The experiments reported here were done in collaboration with Dr. Hadley Kirkman in the Department of Anatomy at Stanford University. In the female hamster, treatment with subcutaneously implanted pellets of diethylstilbestrol for periods averaging about 400 days, brought about uterine hypertrophy with excessive cystic glandular hyperplasia. When testosterone propionate was administered simultaneously, multiple uterine leiomyomas developed. These are apparently histologically identical with tumors reported by Lipschütz in the guinea pig following treatment with estrogens alone. It is of interest that simultaneous administration of testosterone is reported by Lipschütz to protect the guinea pig from the tumors.

In the male reproductive system, estrogen treatment led to severe testicular atrophy, as described for other species, but in addition occasionally proceeded to radical architectural

breakdown with eventual disappearance of the tubular remnants. Four tumors were found resembling the rare "tubular adenoma" of the human testis. Hyperplastic and metaplastic changes in the rete occurred in most treated animals. Other changes were similar to those reported for most mammals. Treatment with both diethylstilbestrol and testosterone propionate resulted in the development of massive leiomyomas of the vas deferens and epididymis histologically indistinguishable from those found in the uterus of similarly treated females.

Of 102 males treated with diethylstilbestrol for 250 days or longer, 98 per cent developed renal mixed tumors, and 46 per cent showed metastases throughout the abdomen. Only 2 animals had extra-abdominal metastases, these in cervical lymph nodes. Tumors do not develop in intact females, but do appear in those which have been ovariectomized. Progesterone or testosterone administered with the estrogen in males or ovariectomized females prevents the development of the tumors.

THE PRESENT RATIONAL TREATMENT OF PORTAL HYPERTENSION

ERIC M. NANSON

Portal hypertension is a serious condition because of hemorrhage from esophageal varices. One hundred cases of portal cirrhosis were admitted to Johns Hopkins Hospital between 1939–1944. Twenty-five of these cases had hematemesis and of these 22 died either in hospital or shortly after discharge. Lord (Rev. Gastroenterology, 1953, **20,** 295) states that 25% of patients bleeding from esophageal varices due to portal hypertension will die from the first hemorrhage, and 70% will be dead within two years.

Portal hypertension is due to obstruction to the outflow of the blood from the splanchnic area. This obstruction may be either prehepatic, intrahepatic, or posthepatic.

Posthepatic obstruction is rare; it produces the Budd-Chiari syndrome, and is not further discussed. Prehepatic obstruction of the portal vein usually occurs in children. The obstruction may be congenital, or may be due to thrombophlebitis secondary to omphalitis in the neonatal period. These children have perfectly normal livers and consequently normal liver function. They develop severe portal hypertension with esophageal varices, and death comes from hemorrhage from these varices. Banti's syndrome and disease are terms applied to this condition. It is, however, urged that these terms be dropped from the literature and the term Extrahepatic Obstruction be substituted, because the fallacious idea still persists that a fibrosis starts in the spleen and subsequently spreads to involve the liver. On these grounds the surgical crime of splenectomy in this condition has been rationalized in the past.

The ideal treatment for extrahepatic portal vein obstruction is splenorenal anastomosis end-to-side. If the spleen has unfortunately been removed previously, then some direct attack on the varices is advocated. Procedures such as gastric transection (Proc. Roy. Soc. Med., 1950, **43,** 147), esophageal transection, or direct ligation of the varices may be used; the object is to tide the patient over until such time as an adequate collateral circulation may open up to reduce the hypertension naturally.

Intrahepatic obstruction is usually due to cirrhosis of the liver and is most frequent in adult life. Because of the liver cirrhosis, the problem of diminished liver function may be added to that of portal hypertension. It is therefore very necessary to assess liver function before surgical treatment is undertaken.

These patients may be classed as follows:

(1) Those with portal hypertension plus good liver function. These should be treated by portacaval anastomosis end-to-side.

(2) Those with portal hypertension plus poor liver function. These shoud be treated

vigorously by medical regime first to endeavor to correct their liver function. If this is accomplished, then they should have a portacaval anastomosis.

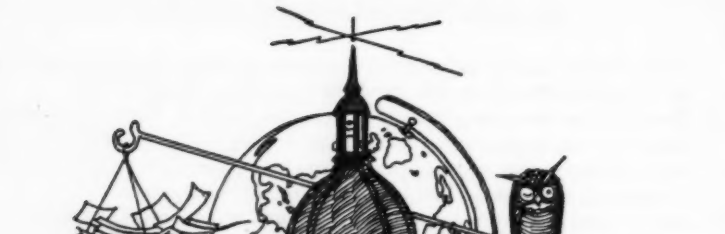
(3) Those with severe hematemesis plus poor liver function. These cases should be treated by emergency direct attack on the bleeding varices to control the hemorrhage. Then vigorous medical therapy should be applied. When the liver function is improved, then a portacaval anastomosis should be performed.

The speaker described the demonstration of the portal venous system by means of injecting a radio-opaque dye, 70% Diodone, into the spleen by percutaneous puncture. Its use in demonstrating the site of the obstruction was stressed, and also its use in showing the patency of a portacaval anastomosis postoperatively.

Blakemore's (Surg. Gyn. and Obst., 1952, **94,** 443) and Linton's (Md. State Med. J., 1953, **2,** 400) figures were quoted demonstrating the value of portal-systemic shunts. A plea was made for a more vigorous and earlier direct attack on the bleeding site in hematemesis from esophageal varices in those patients who were not fit to stand a shunt procedure.

BIBLIOGRAPHY

1. Blakemore, A. H.: Portacaval shunting for portal hypertension. Surg. Gynec. & Obst. 1952, **94:** 443–454.
2. Linton, R. R.: Bleeding esophageal varices and their surgical treatment. Md. State Med. J. 1953, **2:** 400–410.
3. Lord, J. W.: Portal systemic shunts in the management of portal hypertension with massive gastrointestinal hemmorrhage. Rev. Gastroenterology, 1953, **20:** 295–299.
4. Tanner, N. C.: Gastroduodenal bleeding as a surgical emergency. Proc. Roy. Soc. Med. 1950, **43:** 147.



BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of the Bulletin)

Stedman's Medical Dictionary. Eighteenth Revised Edition. Edited by NORMAN BURKE TAYLOR in collaboration with ALLEN ELLSWORTH TAYLOR. 1561 pp., $11.50. The Williams & Wilkins Co., Baltimore, Md.

Although this new edition provides an appreciable increase in word content and in size over its predecessor, it loses none of its chief function as a ready source of reasonably complete word derivation and definition. The addition of several thousand words is sufficient to effect "modernization" of content, as exemplified by inclusion of the newer antibiotics, vitamins and hormones. Attempts are made to limit the increase in size by restricting duplication of entries, by omission of trade names, and by space-saving techniques such as reference to the word being defined by use of its first letter only.

The selection of the 22 plates favors the anatomist particularly, as only six are non-anatomical. Several of the plates (II, VIII) are so exhaustive in detail as to sacrifice clarity. A total of 54 tables is arranged in the context in proper alphabetical order, of which the most are useful and present anatomical terms in the English equivalents of the Latin, which is a desirable practical innovation. Nearly 600 illustrations of variable utility have been added, including a significant number of chemical formulae. A section of medical etymology contains a list of important Greek and Latin words serving as root-words of medical terminology.

Although distinctly uncommon, several errata do appear: the entire caption under Plate XVI reappears erroneously under Plate XXI. The "Diagram of Actions of Ocular Muscles" (after Marquez) on p. 889 shows the oblique muscles producing abduction and the superior and inferior recti performing adduction, which actions are in fact reversed.

The volume exhibits sturdy binding, neat format and handy indexing, and is attractive. It is recommended as a worthwhile addition to the modern medical desk or library.

NORMAN E. SHARRER

Problems of Infancy and Childhood. Transactions of the Sixth Conference March 17 and 18, 1952. Edited by MILTON J. E. SENN. 160 pp., $2.50. The Josiah Macy, Jr. Foundation, New York.

This book is a report of the transactions of the sixth conference sponsored by the Josiah Macy, Jr. Foundation and held on March 17 and 18, 1952. The discussions are centered around the first year of life, covering three main topics: emotional development, individual tendencies, and excessive crying. Besides, there is a brief summary of the activities of the World Health Organization. This reviewer is, as always, impressed by the soundness of Escalona's scientific attitude. One of her remarks is worth quoting and requoting: "What I should like so very much to do is to arouse an interest in looking at what there is to be seen phenomenologicaly before making too explicit certain theoretical conceptions, to look at the data without trying immediately to make it either confirm or deny certain assumptions that one may have." This sentence—and I am afraid, it alone—is worth the price of the book. The somewhat gobbledygookish play on "contagion" versus "resonance," "communication" versus "reaction," or "constitution" versus "equipment," the speculative physiologizing of psychology and psychologizing of physiology, the *ex cathedra* prediction of a "weaning trauma" twenty years hence, and the Talmudic flavor of some of the dialogue tend to confuse rather than clarify. What is one to make of a statement such as this: "The telescope of the mental apparatus fuses a large number of detailed imprints which in the unconscious may all gain the meaning of oral deprivation"? There are more such gems in the book. It is devoutly to be desired that a future historian of present-day studies of infancy and childhood will not use this talkfest as pertinent source material; if he did, his readers might come to underestimate the substantial work that really is being done.

LEO KANNER

Deutsch-Englisches, Englisch-Deutsches Worterbuch fur Arzte. Band 2. By F. LEJEUNE AND W. E. BUNJES. 1737 pp., Ganzleinen DM 58.50. Georg Thieme Verlag, Stuttgart, Germany.

This compact dictionary of medical terms is the companion to a similar volume "German-English Dictionary for Physicians". The format is convenient, and the thin paper and flexible cloth binding of excellent quality. About 75,000 English words are given with accurate German counterparts. This number is large enough to include all terms in general medical use and a great many very rare terms found occasionally in older works. This book constitutes in essence a list of medical terms of Greek and Latin origin with the corresponding words of Germanic origin, for example: "egophony (pronunciation)—Meckerstimme"; "gastroenterostomy (pronunciation)—Gastroenterostomie"; "Anlegen einer Anastomose Zwischen Magen und Darm."

The book may be confidently recommended to those needing a very complete yet pocket-size dictionary of this sort.

DANIEL J. TORRANCE

Physiology of the Eye. Clinical Application, 2nd edition. By FRANCIS HEED ADLER. 734 pp., $13.00. The C. V. Mosby Co., St. Louis, Missouri.

Three years have elapsed since the publication of the first edition of this textbook for students of ophthalmology and practicing ophthalmologists. As the author states the main plan and purpose of this volume continues to be the same, namely, to provide a book which supplies the recent findings of the physiology of the eye gathered from the experimental laboratory and to relate these facts whenever possible with clinical concepts.

The format of the text is unchanged in that the chapters are in the same order and deal with the same subjects. Although the preface states that a considerable amount of new material has been added, in the opinion of the reviewer a moderate amount would more aptly describe the additions, yet this observation does not mean to detract from the great labor required to keep such a textbook up-to-date. Some additions were made to the section on the cornea. In the paragraph added under permeability of the cornea the reference in the bibliography has an incorrect volume number and the page number is reversed. The chapter dealing with the aqueous humor contains new material on the passage of test substances across the blood-aqueous barrier. Important recent experiments determining the facility of outflow of aqueous humor in normal and glaucomatous eyes have been added to the section on the intraocular pressure. To the chapter on the lens and vitreous has been added information about the lens capsule, the enzyme systems responsible for lens respiration, and the fibrous components of the vitreous body which have been examined with the phase and electron microscope. The section on ocular circulation has been amplified by including material on the structure and probable function of the aqueous veins. The chapter on ocular motility is an outstanding discussion of the subject. On page 565 the reference to figure 146, page 388, should be to figure 145, page 387. The chapter on visual acuity was partly rewritten with new material as well as additional figures and contains several examples whereby the author's knowledge of ocular physiology is used to explain clinical observation or findings.

This book is, and deserves to be, the standard American text on the physiology of the eye.

W. G. MARR

The Unique Influence of The Johns Hopkins University on American Medicine. By RICHARD H. SHRYOCK. 77 pp., Danish kr. 14.00 (bound) ($2.18). Ejnar Munksgaard. Copenhagen, Denmark.

The influence on American Medicine that is described in this monograph is indeed "unique". Dr. Shryock has presented in a most stimulating manner the idealism, vision, enthusiasm and energy that have contributed to and emanated from The Johns Hopkins Medical Institutions. The contributions and roles of Osler, Welch, Halsted, Kelly, Billings, Hurd and many others are woven into the fabric of the story. It is the story of original concepts and developments of new patterns of medical education, training, research and patient care. The value of such innovations has been attested partially by the later activities and contributions of former students and associates of The Johns Hopkins University.

This monograph should be carefully studied by all who are interested in the past and future of American Medicine.

PHILIP F. WAGLEY

BOOKS RECEIVED FOR REVIEW

Administrative Medicine. Transaction of the First Conference March 9, 10 and 11, 1953. Edited by GEORGE S. STEVENSON. 176 pp., $3.00. Josiah Macy, Jr. Foundation, New York.

Alfred Hospital Clinical Reports, Volume 3. Edited by R. S. LAWSON and Committee. 115 pp. Brown, Prior, Anderson Pty. Ltd., Melbourne, Australia.

Arzt und Neurose. By J. H. SCHULTZ. 107 pp., kartoniert DM 8.40. Georg Thieme Verlag, Stuttgart, Germany.

The Child, His Parents and the Nurse. By FLORENCE G. BLAKE. 440 pp., $5.00. J. B. Lippincott Co., Philadelphia, Pa.

Connective Tissues. Transactions of the Fourth Conference February 18, 19 and 20, 1953. Edited by CHARLES RAGAN. 197 pp., $3.75. Josiah Macy, Jr. Foundation, New York.

Intensive Group Psychotherapy. By GEORGE R. BACH. 446 pp., $6.00. The Ronald Press Co., New York.

An Introduction to Human Biochemical Genetics. By H. HARRIS. 96 pp., $2.75. Cambridge University Press, New York.

Klinische Zytologie. Grundriss der Allgemeinen Zytologie und der Zytodiagnostik. By H. J. STREICHER AND ST. SANDKUHLER. 196 pp., Ganzleinen DM 49.80. Georg Thieme Verlag, Stuttgart, Germany.

Lehrbuch der Gynäkologie. By HEINRICH MARTIUS. 414 pp., Ganzleinen DM 49.80. Georg Thieme Verlag, Stuttgart, Germany.

Le Leptospirosi. By MARIO AUSTONI. 715 pp. Isituto di Pathologia Speciale Medica E di Metodologia Clinica, Dell 'Universita di Padova.

Medical History of the Second World War. Surgery. Edited by SIR ZACHARY COPE. 772 pp., 80s. Her Majesty's Stationery Office, London, England.

Medizin in Bewegung, 2nd edition. Klinische Erkenntnisse und Arztliche Aufgabe. By RICHARD SIEBECK. 496 pp., Ganzleinen DM 33. Georg Thieme Verlag, Stuttgart, Germany.

Michael Servetus. A Translation by CHARLES DONALD O'MALLEY. 208 pp., $3.00. The American Philosophical Society, Independence Square, Philadelphia, Pa.

Music Therapy. Edited by EDWARD PODOLSKY. 335 pp., $6.00. Philosophical Library, New York.

Plant Proteins in Child Feeding. Medical Research Council Special Report Series No. 279. By R. F. A. DEAN. 164 pp., $2.25. Her Majesty's Stationery Office, London, England.

Principles of Internal Medicine. By T. R. HARRISON, P. B. BEESON, W. H. RESNIK, G. W. THORN AND M. M. WINTROBE. 1617 pp., $15.00. The Blakiston Co., New York.

Traumatismos Medulares. Clinica Y Tratamiento. By VICTOR SORIANO. 357 pp. Editorial Labor, S. A. Argentina. Buenos Aires, Montevideo.

Unternehmer Krankheit. Entstehung und Verhütung. By M. HOCHREIN AND I. SCHLEICHER. 60 pp., kartoniert DM 4.80. Georg Thieme Verlag, Stuttgart, Germany.